Cape Peninsula University of Technology

# The effects of kolaviron on epididymal and testicular function in streptozotocin induced diabetic wistar rats

**By**

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## DECLARATION

I, Claudine Manirafasha, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed Date

## ABSTRACT

Oxidative stress (OS) plays a central role in the progression of diabetes mellitus (DM). Prevention of DM and its complications is a challenging health problem as it impacts on various organ functions, including reproduction. Diabetes mellitus with hyperglycaemic condition generates high production of reactive oxygen species. An imbalance between antioxidant mechanism and reactive oxygen species generates oxidative stress. OS damages the sperm membrane by oxidation of polyunsaturated fats which in turn reduces the sperm motility and ability to fuse with the oocyte and OS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo development.

Recent experimental evidence shows that modulation of oxidative stress and natural antioxidants may determine the outcome of male reproductive function. Previous investigations indicate that the supplementation and treatment with phytomedicine might play role in the prevention and management of DM and its subsequent complications on male reproductive function. This study explored the pharmacological potential of kolaviron (KV) on testicular and epididymal tissue in diabetic and non- diabetic Wistar rats. All experiments were conducted for a period of six weeks. Male Wistar rats (240–290 g) were randomly divided into 5 groups (n=12) where all the rats received a standard diet. Non diabetic rats control group and other four groups injected with different treatments. Non diabetic rat (N) received vehicle: Dimethylsulfoxide. Diabetes rats (D) were induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ) solution, 50mg/kg body weight. The N and D were treated with kolaviron (100 mg/kg body weight) orally, five times a week .The last group, diabetic rats were given subcutaneously injection of the standard anti-diabetic drug, insulin (0.2 u/kg) every second day. After the feeding period, testicular and epididymal tissues were collected and were analysed. All parameters were determined using appropriate methods in homogenized tissues. Data were expressed as mean ± SD.

Plasma glucose as well as malondialdehyde (MDA) was significantly higher, while body, testicular and epididymal weights were lower in the D group compared to the N group and N+KV. Both kolaviron and insulin were able to ameliorate these effects.

Testicular and epididymal antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in induced diabetic rats were significantly ($p<0.05$) low compared to diabetic control group. However, KV treated group shown significantly higher SOD, CAT and GPx activities compared D group.

In conclusion, our findings demonstrated that KV could improve antioxidant enzymes and modulate STZ induced diabetic related oxidative stress in the male reproductive system. Kolaviron can potentially be used as an anti-diabetic treatment, however further studies are needed.

**Key words:** Oxidative stress, Diabetes mellitus, antioxidants, kolaviron, epididymal tissue, testicular tissue, superoxide dismutase, catalase, glutathione peroxidase, lipid peroxidation, streptozotocin

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## DEDICATION

To my beloved Parents:

Fabien Bizimungu and Laurence Mukambuguje,

To my child

Michel Archange Mugisha Nshimiyimana

## LIST OF OPERATIONAL TERMS AND CONCEPTS

| | |
|---|---|
| **Antioxidant** | A potential scavenger of reactive oxygen species |
| **Diabetes** | Uncontrolled blood sugar |
| **Epididymal function** | maturation and temporal storage of spermatozoa |
| **Free radical** | Any compound which contains one or more unpaired electrons |
| **Garcinia kola** | Medicinal plant from Guttiferae family |
| **Hyperglycaemia** | Increased blood sugar due to decreased insulin secretion or insulin resistance |
| **Infertility** | State of being unable to produce offspring after twelve mouths of unprotected intercourse |
| **Insulin** | Endocrine hormone secreted by pancreas |
| **Kolaviron** | Bioflavonoids from Garcinia kola |
| **Lipid peroxidation** | Oxidation of polyunsaturated fats |
| **Oxidative stress** | Cellular condition associated with an imbalance between the production of free radicals and the antioxidant mechanisms. |
| **Reactive oxygen species** | Highly reactive oxidizing agents belonging to free-radicals |

| | |
|---|---|
| **Streptozotocin** | Antibiotic with diabetogenic effects |
| **Testicular function** | Production of testosterone and spermatozoa |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AKT | Protein kinase |
| ºC | Degrees Celsius |
| CAT | Catalase |
| DNA | Deoxyribonucleic acid |
| GPX | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Reduced glutathione |
| GSSG | Oxidised glutathione |
| HPLC | High performance liquid chromatography |
| $H_20_2$ | Hydrogen peroxide |
| KV | Kolaviron |
| LPO | Lipid peroxidation |
| mg | Milligram |
| min | Minute |
| Ml | Millilitre |
| MDA | Malondialdehyde |
| µmol | Micromole |
| NAD(P)H | Nicotine adenine dinucleotide phosphate |
| $NO^{-}$ | Nitric oxide |
| $O_2$ | Oxygen |
| $O_2^{\cdot -}$ | Superoxide |
| $ONOO^{-\cdot}$ | Peroxynitrite |
| Ox-LDL | Oxidized low-density lipoprotein |
| ROS | Reactive oxygen species |
| SOD | Superoxide dismutase |
| SRC | Standard rat chow |

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# CHAPTER ONE

# INTRODUCTION

## 1.1Background

The World Health Organization defines infertility as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after twelve months or more of regular unprotected sexual intercourse (WHO, 2010). Male factor infertility can contribute between 30-50% to this condition and may result from several factors such as physiological, systemic pathologies, genetic abnormalities, environmental pollution and oxidative stress (Oelmedo *et al.,* 2005; Maneesh *et al*., 2006).

Oxidative stress (OS) is described as an imbalance between the production of reactive oxygen species (ROS) and their removal or reducing agents called antioxidants (Makker *et al*., 2009). This state of OS potentially leads to the damage of biomolecules such as proteins, nucleic acids and lipids (Uttara *et al*., 2009). In recent years OS has become more prevalent and has significantly contributed to abnormal sperm morphology (Guz *et al*., 2013; Taib *et al*., 2013; Beloc *et al*., 2014; Lee *et al* ., 2014 ), sperm quality and quantity (Herwig *et al*., 2013).

Epidemiological and experimental studies have indicated a potential relationship between OS and male infertility (Doreswamy *et al.,* 2004; Albert *et al*., 2013). In the testes, OS is capable of disrupting the steroidogenic capacity of Leydig cells as well as the spermatogenesis process (Hales *et al.*, 2005). Spermatozoa contain polyunsaturated fats (PUFAs), limited cytoplasm antioxidant enzymes (Aitken *et al*., 2007) and are susceptible to oxidative attack. The free radical attack can induce lipid peroxidation (LPO) and DNA fragmentation; disrupting both sperm development and motility (Aitken *et al*., 2007; Kefer *et al.,* 2009).

The WHO reported (2010) that male infertility is commonly due to deficiencies in the semen volume, sperm concentration and sperm quality. These deficiencies often cause abnormalities such as azoospermia (absence of spermatozoa), oligozoospermia (decreased number of spermatozoa), asthenozoospermia (decreased sperm motility), teratozoospermia (abnormal sperm morphology) and or

a combination of the three conditions (oligo-astheno-teratozoospermia) (Dohle *et al*., 2005).

Almost 50 million couples worldwide are unable to have a child after five years of trying (Maya *et al*., 2012). This may result from infertility from either one or both partners (Oelmedo *et al*., 2005). According to Dohle and co-workers (2005), about 25% of couples do not achieve a pregnancy within 1 year, 15% of the couples seek medical treatment for infertility and ultimately less than 5% remain childless. Sharlip *et al.* (2002) argued that infertility affects approximately 15 % of couples, attempting pregnancy (in US) and among which half can be attributed to male factor. In addition, there is a worldwide rise in incidence of diabetes mellitus (DM) which can be associated with the increasing prevalence of infertility in men of reproductive age (Agbaje *et al*., 2007; Mallidis *et al*., 2007).

Diabetes mellitus is a group of metabolic conditions which are characterised by high glucose levels (hyperglycaemia) caused by abnormal insulin secretion/insulin deficiency, abnormal insulin action or both. Classically there are 2 main types of DM: Type 1 diabetes mellitus (T1DM) and Type 2 diabetes mellitus (T2DM). The T1DM is the consequence of insulin deficiency while T2DM is characterized by insulin resistance. It has been demonstrated that diabetes has a direct effect on male fertility (Mallidis *et al*., 2007). About 90% of diabetic patients have disturbances in fertility characterized by a decreased semen volume, sperm count, motility and morphology (Ramalho-Santos *et al*., 2008), increased seminal plasma abnormalities, and reduced serum testosterone levels because of impaired Leydig cell (Amaral *et al.,* 2008).

The effect of OS induced by DM in the male reproductive system *in vivo* is well established (Shrilatha and Muralidhara, 2007). Infertility and subfertility prevalence is high in male patients with T1DMand T2DM (Delfino *et al*., 2007). Moreover, data from animal models strongly suggest that DM impairs male fertility at multiple levels, such as endocrine control of spermatogenesis, spermatogenesis itself, or by impairing penile erection and ejaculation (Cameron *et al*., 1990; Ballester *et al*., 2004; Scarano *et al*., 2006). Nevertheless, although the problems arising from DM have been widely investigated, the mechanisms responsible for the reported male

reproductive dysfunction are still poorly understood (Agbaje *et al*., 2007; La Vignera *et al.,* 2011). Recent reports have highlighted changes in the whole body metabolic profile in diabetic conditions (Salek *et al*., 2007; Zhao *et al*., 2010), though only one has reported on the testicular metabolic profile under the diabetic state (Mallidis *et al*., 2009). The detrimental influence of DM on testicular metabolism is receiving increased attention and recently it was suggested that testicular cells of diabetic individuals may present metabolic adaptations that allow them to minimize the negative effects promoted by the disease (Amaral *et al*., 2006). Hence the search for different treatment options in male oxidative infertility have been proposed including assisted reproduction, reproductive surgery, nutritional advice (derivative of plant material), fertility counselling and fertility acupuncture (Zhu *et al*., 2006).

Studies using several experimental investigations have demonstrated the potential of antioxidant rich supplements in the management of induced oxidative stress-induced in animal models (Ross *et al*., 2010). However, the use of derivatives of plant materials might be important and effective because they are less toxic, affordable as well as minimises side effects or risks caused by other options (Larkins and Wynn, 2004). The Phytochemicals are considered strong natural antioxidants and have an important role in health care systems (Temple, 2000). They possess adaptatogen characteristics, able to respond to stress and help regulate the interconnected endocrine, immune, and nervous systems (Mahajan *et al*., 2012).

The *Garcinia kola (G kola)* nut, commonly referred to as kolaviron (KV), has shown great potential for use in therapeutic medicine against many health threatening chronic diseases of the liver, reproductive system and diabetes (Farombi *et al*., 2004a; Farombi *et al*., 2004b; Adaramoye, 2010; Farombi and Owoeye, 2011). It is widely used in traditional medicine in southern Nigeria for the treatment of different conditions associated with increased OS (Farombi *et al.,* 2004a). Kolaviron is known to possess antihyperglycaemic effects in normal, alloxan and streptozotocin (STZ) induced diabetic animals (Iwu *et al*., 1990; Adaramoye and Adeyemi, 2006). Moreover, KV also elicits strong antioxidant activity in *in vivo* and *in vitro* models (Adaramoye *et al*., 2005). This property is due to the high flavonoid (biflavanoids) contents which are able to terminate the free radical chain reactions in response to OS (Adaramoye *et al.,* 2005; Oluwaseun and Ganiyu, 2008). This is important as it

will shed light on the natural antioxidant properties of KV which is an important consideration in the design of new treatment regimens for male infertility due to OS.

## 1.2 Aim and Objectives

The aim of this study was to investigate the effect of KV on testicular and epididymal tissue in diabetic and non-diabetic rats. The specific objectives of the study were:

- To determine if STZ could induce diabetes associated with OS in rat;
- To investigate whether KV treatment can reduce, moderate, inhibit or reverse the induced OS effects in testicular and epididymal tissues;
- To investigate whether KV treatment can increase production of testicular and epididymal antioxidant enzymes;
- To investigate the effects of KV treatment compared to the standard treatment using insulin in STZ induced diabetic epididymal and testicular tissues.

# CHAPTER TWO

# LITERATURE REVIEW

Mammalian spermatozoa, like other cells living under aerobic conditions, constantly use oxygen for supporting life (Metcalfe and Alonso-Alvarez, 2010). It is found that 1-5% of all inhaled molecular oxygen ($O_2$) during mitochondria respiration highly reacts with other molecules to generate downstream oxygen derivatives called ROS and reactive nitrogen species (ROS/RNS) (Cann and Hughes, 2002; Berk, 2007; Metcalfe and Alonso-Alvarez, 2010). Oxygen is very important for cellular metabolism to produce energy. This is achieved through oxidation of organic substances (Metcalfe and Alonso-Alvarez, 2010).

Reactive oxygen species are chemically reactive molecules containing $O_2$ and represent a broad category of molecules, including a collection of radical (hydroxyl ion ($OH^-$), superoxide ion ($O_2^-$), nitric oxide($NO^-$), and non-radical ozone($O_3$), singlet oxygen($O_2$) , lipid peroxide($LOO^-$), hydrogen peroxide($H_2O_2$) are oxygen derivatives (Agarwal *et al*., 2005). Oxygen derivatives are formed in different reactions and become reactive (Halliwell and Gutteridge, 1999). Superoxide and peroxide molecules are formed respectively from the reduction reaction of oxygen; the hydroxyl radical is produced by reaction between superoxide and hydrogen peroxide or reduction of peroxide catalysed by ferrous ions (Ford, 2004). Superoxide reacts with nitric oxide to form peroxinitrite, and hydrogen peroxide can react with chloride to form hypochlorite. Reactive nitrogen contains nitrogen and derived from nitric oxide and superoxide.

Reactive species have been described to show both biopositive and bionegative effects (Valko *et al.*, 2007). In the normal state, when ROS/RNS are low in concentration, there is a minimal oxidative damage which contributes to physiological signalling through redox reaction (Lavrovsky *et al.,* 2000). However, it could be said that diseases and environmental factors contribute to excess ROS/RNS production resulting in oxidative damage (Lavrovsky *et al.,* 2000; Covarrubias *et al.,* 2008). In a situation where the reactive species are produced more than antioxidants, OS occurs (Figure 2.1). Under OS conditions, cellular

components are damaged and various disorders in organism are evident (Lin and Beal, 2006; George and Ojegbemi, 2010; Galley, 2011; Pirinccioglu *et al.,* 2012).

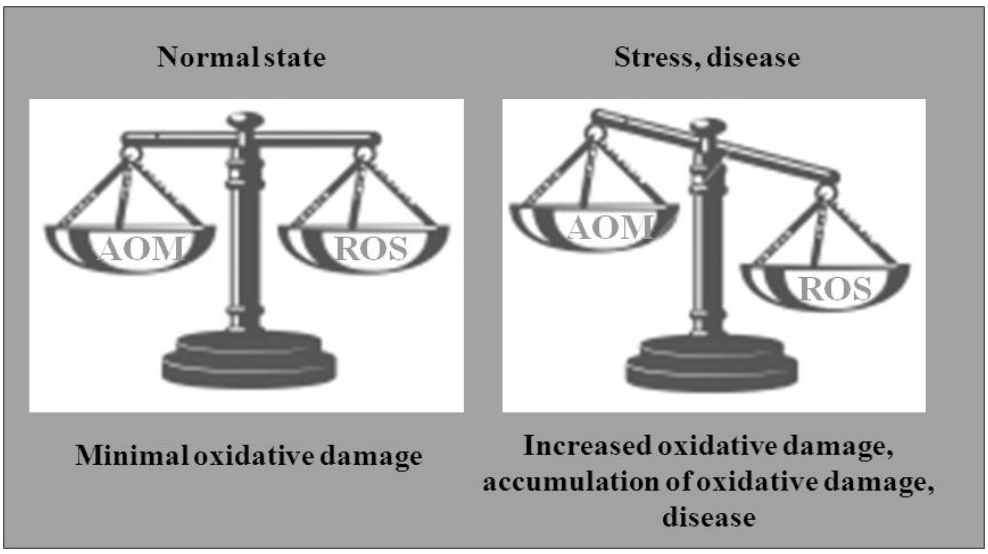


**Figure 2. 1: Schematic drawing of the metabolic balance and imbalance related to oxidative stress (Adapted from Covarrubias *et al.,* 2008)**
*Abbreviations:* AOM: Antioxidant molecules, ROS: Reactive oxygen species

## 2.1. Sources of reactive oxygen species in the male reproductive system

The oxygen derivatives in the male reproductive system are produced both exogenously and endogenously by enzymatic and non-enzymatic activities (Rahman, 2007). They play an important function depending on their nature and concentration in spermatozoa (Zelen *et al*., 2010). Superoxide anion, hydroxyl radical and hydrogen peroxide are the highly reactive metabolites of oxygen and exist in the seminal plasma (Agarwal and Prabakaran, 2005).

### 2.1.1 Internal sources of reactive oxygen species

Endogenous ROS are produced in the mitochondria as by-products of normal cellular respiration and their increase is related to defective oxidative phosphorylation (Kirkinezos and Moraes, 2001). Leukocytes and spermatozoa have been shown to be the two main sources of ROS (Garrido *et al.,* 2004). Moreover,

male infertility has been linked with increased ROS production by immature, abnormal spermatozoa and by contaminating leukocytes (Maneesh and Jayalekshmi, 2006).

Spermatozoa are male reproductive cells produced by the testis during the process of spermatogenesis. Depending on the species, intrinsic and extrinsic factors, spermatogenesis takes place through different stages at precise times (Hess and França, 2007). For example, the total duration of spermatogenesis is at least 40-54 days in the rat and 70- 74 days in men (França and Russell, 1998; Hess and França, 2007). Spermatozoa are able to generate ROS; however it is dependent on the maturation level of the spermatozoon (de lamirande and lamothe, 2010). During the differentiation of spermatocytes into mature spermatozoa, they have the potential to generate ROS (Agarwal *et al.,* 1992; Agarwal *et al.,* 1994; Dacheux, 2003). The ROS production depends on two mechanisms; nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system of the sperm plasma membrane (Aitken *et al*., 1992), and NADPH-dependent oxidoreductase in the mitochondria (Banfi *et al*., 2001). Other ROS generated in spermatozoa include lipoxygenase and cytochrome P450 reductase (Baker *et al*., 2004). In addition, immature and abnormal spermatozoa resulting from impaired spermatogenesis contribute in the regeneration of ROS (Thomas and Joseph, 1994; Saleh *et al*., 2002). Moreover, these cells with retained cytoplasmic residues create intrinsic OS because of high levels of the cytoplasmic enzymes. These cytoplasmic enzymes include glucose-6-phosphate dehydrogenase (G-6-PDH), creatine kinase and lactic acid dehydrogenase (LDH) which generate NADPH as source of ROS via NADPH oxidase (NOX) activity (Henkel, 2005; Said *et al*., 2005).

Leukocytes such as neutrophils and macrophages are present mostly throughout the male reproductive tract and are found in almost every human ejaculate (Sharma *et al*., 2001). It has been suggested that leukocytes have the physiological role of removal of dead or abnormal spermatozoa in semen (Tomlison *et al*., 1993). During infection and inflammation of tissue; leukocytes respond by generating and releasing high concentrations of ROS to form cytotoxic reactions against nearby cells and pathogens (Kumar, 2007). These phagocytic leukocytes contain a NADPH oxidase which secretes superoxide into the phagocytic vesicle during this digestive reaction

(Vernet *et al*., 2004). The principal enzyme, termed gp91phox, contains a flavoprotein and a cytochrome b558 component and is activated when combined with regulatory subunits (Segal and Abo, 1993; Babior *et al*., 1997; Babior, 1999). It has become clear that gp91phox is a member of a NOX enzyme family widely distributed in other cell types and involved in generating ROS for regulatory and other purposes (Lambeth *et al*., 2000).

De Lamirande *et al*. (1997) have reported the inter-relationship between non-activated leucocytes, activated leukocytes and spermatozoa in terms of ROS production. Activated leukocytes are able to produce 100-fold higher amounts of ROS than non-activated leukocytes and produce 1000-times more ROS than spermatozoa. Superoxide anion and $H_2O_2$ are produced in semen and cause sub-fertility or even infertility in patients (Griveau and Le Lannou, 1997; Henkel *et al*., 2005). However, it has been shown that ROS production by human spermatozoa and contaminating leukocytes can be stimulated by phorbol esters and certain formyl peptides with deleterious effects on sperm motility and male fertility (Pasqualotto *et al*., 2000).

It has been found that an accumulative infiltration of leukocytes in the semen (leukocytospermia) increase interleukin 8 (IL-8), induce ROS production, and decrease SOD activity (Ochsendorf *et al*., 1999). This demonstrates that increased OS during leukocytospermia is caused by a defective ROS scavenging system, which, in turn, can be modulated by certain pro-inflammatory cytokines. A significant shift towards increased production of pro-inflammatory chemokines (GRO-a) compared to anti-inflammatory cytokines (IL-10) during leukocytospermia suggests an active chemotactic pro-inflammatory response (Sharma *et a*l., 2001). This shift may be responsible for the acute OS condition observed in the semen of infertile individuals (Sikka *et al*., 1996).

### 2.1.2 External sources of ROS

Exogenous sources of ROS include chemical compounds as well as pathologies and systemic infections (Park *et al.,* 2003). External sources increase ROS and impair

the function of the male reproductive organs thereby decreasing fertility (Cocuzza *et al.*, 2007).

Radiation is a natural energy source with significant clinical effects on virtually every living organism, including humans. A recent study on the potential ability of ionizing radiation to cause ROS production in sperm has been conducted in South Korea (Kim *et al*., 2011). In this original research study, scientists exposed mice to pelvic irradiation using a 5 Gray source to determine its effects on sperm production and function. In the control group, testicular apoptosis was largely induced, affecting both germ and Sertoli cells and leading to decreased sperm cell counts and subnormal motility indexes (Kim *et al*., 2011). Moreover, the irradiated animals produced sperm with particularly increased ROS concentrations, which could account for the observed structural and functional abnormalities of the spermatozoa (Kim *et al*., 2011).

Additionally, the recent increase in mobile phone distribution and use has motivated the research to investigate radiofrequency electromagnetic wave (RF-EMW) radiation of cell phones (Ozguner *et al*., 2006; Balci *et al*., 2007). Several males carry mobile phone near or close to their genital organs. As a result, there is also now ample evidence on the *in vivo* effect of prolonged exposure of the testis to mobile phone radiation (Aitken *et al*., 2005; Agarwal *et al*., 2008). Numerous *in vivo* animal studies have specifically revealed the induction of OS as a result of RF-EMW radiation of cell phones (Ozguner *et al*., 2006; Balci *et al*., 2007). Within the context of male reproductive health, several studies have also suggested that mobile phone radiation can increase the production of ROS in human semen (Erogul *et al*., 2006; Agarwal *et al*., 2008; De Iuliis *et al*., 2009). A potential pathway via which ROS production can occur after exposure to RF-EMW radiation by stimulation of spermatozoa plasma membrane NADH oxidase (Agarwal *et al*., 2008). Alteration of NADH oxidase function can lead to OS by increasing the production of ROS (Friedman *et al.,* 2007).

The potential toxicity of medications in testicular function and sperm production are well documented. Such medications include cyclophosphamide, aspirin paracetamol, tetracyclines, aminoglycosides and chloramphenicol have been reported to increase

ROS production thereby causing OS (Das *et al.*, 2002; Ghosh *et al*., 2002; Agarwal and Said, 2005; Farombi *et al*., 2008; Naravana, 2008; Ovagbemi *et al.,* 2010). For instance, injection of cyclophosphamide in animals enhanced the production of MDA as a product of LPO and inhibited the testicular CAT activity (Das *et al.*, 2002; Ghosh *et al*., 2002). Aspirin and paracetamol treatment have shown to increase cytochrome P450 activity which is a main source of ROS (Agarwal and Said, 2005).

A number of chemical toxins such as ethylene glycol and its byprod-ucts, bisphenol A, dioxin and phthalates can have a negative impact on sperm structure and function (Adedara *et al*., 2010; Lavranos *et al*., 2012). These substances may be released from structural materials, industrial products or other consumables and tend to accumulate in the human body, causing long-term adverse effects due to chronic intoxication (Makker *et al*., 2009). Toxin substances have been shown to increase LPO and decrease antioxidant enzymes activity; subsequently, they might cause low fertility (Lavranos *et al*., 2012).

Spermatogenesis disruption and DNA sperm damage have been reported in smokers (Kothari *et al*., 2010). More than 4000 chemicals in addition to nicotine in cigarette cause ROS production by suppressing the seminal antioxidants activities (Traber *et al*., 2000; Zenzes *et al*., 1999). Smoking increases seminal leucocyte concentration which in turn increases ROS in the male reproductive system, thereby leading to the decrease of sperm parameters such as sperm motility, morphology and concentration in smokers (Saleh *et al*., 2002).

Numerous disorders of the male reproductive system such as, cancer, varicocele , cryptorchidism, testicular torsion of the spermatic cords, and diabetes have been associated with male infertility due to OS caused by the uncompensated hyperproduction of ROS (Aitken and De Iuliis, 2007; Tremellen, 2008).Testicular cancer may disturb hormone levels and induces OS (Sharma *et al*., 2004). These have negative effect on spermatogenesis and sperm DNA (Meirow and Schenker, 1995; Thomson *et al*., 2002; Agarwal and Allamaneni, 2004; Van Carsteren *et al*., 2009). Dysgenesis syndrome which is abnormalily inthe male reproductive tract has been indicated in cancer cell of testes (Hoei Hansen *et al*., 2003).

Testicular torsion is a relatively common, painful condition that must be treated rapidly if the testes are not to suffer permanent damage. Prolonged torsion leads to testicular ischaemia and high levels of OS in the ipsilateral testes associated with NO and $H_2O_2$ production, increased lipid peroxide formation, isoprostane accumulation, antioxidant enzyme depletion and increased rates of mitochondria mediated apoptosis in the germ line (Chaki *et al*., 2003; Lysiak *et al*., 2007). Even short periods of ischaemia, for 3 hours or less, can lead to a high level of OS in the testes, depletion of testicular glutathione levels and the consequent disruption of spermatogenesis. Moreover, men with spinal cord injuries tend to have increased levels of ROS and ROS-induced DNA damage specifically because they are prone to genitourinary tract infections (Oliva, 2006; Brackett *et al*., 2008).
Cryptorchidism is infectious disease attacks testicular exposure to high temperature and is associated with oxidative stress resulting from a reduction in SOD and catalase activities (Ahotupa *et al*., 1992). Elevated temperature has been found to induce high rates of apoptosis of spermatogenic cells via mechanisms that were associated with elevated levels of $H_2O_2$ generation (Ikeda *et al*., 1999).

Varicocele is the impaired of testicular venous and is also associated with the disruption of spermatogenesis via mechanisms involving the induction of OS (Marmar *et al*., 2001; Schoor *et al*., 2001). It has also been reported to affect leydig cell and androgen production (Kim and Goldstein, 2008). About 15% of adult males are believed to either have clinical or subclinical varicocele, with a prevalence as high as 40% in infertile males (Schoor *et al*., 2001). In clinical studies, the presence of a varicocele has been shown to correlate with excess ROS generation by the spermatozoa, high rates of DNA damage in these cells and depleted antioxidant levels in the seminal plasma (Smith *et al*., 2006).

Experimental induction of diabetes in animal models has been shown to impair both testicular and epididymal function thereby decreasing male fertility (Aitken and Roman, 2008; Nelli *et al*., 2013). Thus, diabetogens such as streptozotocin enhance ROS generation and induce both sperm lipid peroxidation and protein carbonyl expression in the testes (Amaral *et al*., 2009). Furthermore, diabetic condition with OS caused DNA damage and apoptosis of spermatozoa (Aitken and De Iuliis, 2009).

## 2.2 Reactive oxygen species in male reproductive system

### 2.2.1 Reactive oxygen species and sperm function

The physiological function of radicals in the cells is achieved only if there is a balance between the production and degradation of ROS (Agarwal *et al*., 2006). Agarwal and co-workers (2006) had reported the correlation exists between ROS, sperm concentration, motility, and morphology. At low concentrations reactive oxidants have a biopositive effect and act selectively (Allen and Tresini, 2000, De Lamirande *et al.*, 2009). Aitken and coworkers (1993) reported that a low concentration of hydrogen peroxide did not have any effect on sperm motility, but did suppress sperm-egg fusion (Aitken et al., 1993). This may also explain why patients with normal semen parameters can still experience infertility. In such patients, the ROS levels are not high enough to impair basic semen analysis parameters, but can cause defects in other processes that are required for fertilization, such as sperm-oocyte interaction. Reactive oxygen species also act on the metabolism of prostanoids, in gene regulation or in the regulation of cellular growth, intracellular signalling, development of spermatozoa and fertilization (Allen and Tresini, 2000, De Lamirande *et al.*, .2009). Controlled amounts of $H_2O_2$ and superoxide ions concentration are important for modulating the acquisition of fertilizing ability always involves a successive process including sperm maturation, capacitation, hyperactivation, acrosome reaction, and sperm oocyte fusion (Naz and Rajesh, 2004; Sa´nchez *et al*.,2010).

#### 2.2.1.1 Sperm maturation

Spermatozoa obtained from the testis are unable to exhibit progressive motility or to capacitate, but acquire these abilities during their passage through the epididymis (Gadella *et al*., 2001). Sperm maturation is referred as dynamic morphological and metabolic changes that lead to formation of active spermatozoa able to fertilize ovum (Cooper, 1995). These processes are referred to as maturation. Other maturational changes include the completion of nuclear condensation and changes in the

expression and distribution of molecules on the sperm surface (Cooper, 1995; Moore, 1996; Ford, 2004).

In the sperm nucleus, phospholipid hydroperoxide glutathione peroxidase (GPx4) can utilise the thiol groups in nuclear proteins as an alternative reductant to glutathione. Generation of lipid peroxides by ROS might provide a substrate for GPx4 to drive the oxidation of these proteins and to facilitate nuclear condensation, while simultaneously providing protection against oxidative DNA damage (Pfeifer *et al*., 2001). Reactive oxygen species might also be involved in motility initiation by enhancing cyclic adenosine monophosphate (cAMP) synthesis and protein phosphorylation at the time of ejaculation (Aitken *et al*., 2004).

#### 2.2.1.2 Capacitation

Capacitation is a morphological change that spermatozoa undergo to acquire the ability to fuse with an ovum through series of hyperactivation and acrosome reaction (Yanagimachi, 1994; Gaboriau *et al*., 2007). Hyperactivation of sperm motility is characterised by high amplitude, asymmetrical beating pattern of the sperm tail and allows the spermatozoa to penetrate the zona pellucida of the ovum. It is followed by the acrosome reaction where the head of the mature spermatozoa releases acrosin and other enzymes to digest the cumulus cells and break through the zona pellucida (Gadella *et al*., 2001) (Figure2.2). Studies have shown that $O_2^-$ serves an extremely important regulatory role in facilitating both hyperactivated movement and the induction of the acrosome reaction (de Lamirande *et al*., 1993; Griveau *et al.*, 1997; Flesch and Gadella, 2000; Gadella *et al*., 2001, Ferramosca *et al*., 2013; Ferramosca and Zara, 2014.).

Capacitation is characterized by increased membrane fluidity, increased tyrosine phosphorylation, increased pH levels, increased intracellular cAMP, and calcium influx (Schoeller *et al*., 2012). Capacitation can be controlled by substances present in semen (Fraser and Adeoya-Osiguwa, 2001), by progesterone and other substances secreted by the oocyte cumulus complex (Blackmore, 1993), but can also occur spontaneously under suitable conditions *in vitro* (de Lamirande *et al*.,

1997). Moreover, ROS generated by mammalian spermatozoa may play a physiologically important role in driving the complex process of capacitation through the redox regulation of tyrosine phosphorylation (Leclerc *et al*., 1997; Lewis and Aitken, 2001; Baumber *et al.*, 2003; Ecroyd *et al*., 2003; Rivlin *et al.,* 2004; Thomson *et al*., 2009). The mechanisms supporting this redox effect on protein tyrosine phosphorylation are complex and involve the stimulation of cAMP generation inhibition of tyrosine phosphatase activity, and modulation of a variety of additional signal transduction cascades, including sarcoma- and extracellular signal-regulated kinase mediated pathways (Baker *et al.,* 2004). Therefore capacitation takes place by increasing of membrane fluidity, cholesterol efflux, ion fluxes resulting in alteration of sperm membrane potential, increased tyrosine phosphorylation of proteins, induction of hyperactivation and the acrosome reaction (de Lamirande *et al.*, 1993; Naz and Rajesh, 2004; Sa´nchez *et al*., 2010) (Figure 2.2).

Reactive oxygen species act together with other factors including bicarbonate, loss of membrane cholesterol and increasing intracellular $Ca^{2+}$ leading to activation of adenylyl cyclase (AC) resulting in cAMP production and protein kinase A (PKA) activation and protein tyrosine phosphorylation (Breitbart, 2002). Lewis and Aitken (2001) proposed that superoxide is the species responsible for activating adenyl cyclase, whereas Rivlin *et al.* (2004) suggested that hydrogen peroxides responsible and can substitute for bicarbonate in activating the cyclase. Increased cAMP activates PKA that in turn activates tyrosine kinase (TK) and inhibits tyrosine phosphatase (TP) through unknown mechanisms. The involvement of PKA has been confirmed with the inhibitor of PKA (H89). Hydrogen peroxide directly activates TK and inhibits TP. The increase in tyrosine phosphorylation produced by these changes is the major driving force for capacitation and conduct to hyperactivation, zona binding and acrosome reaction that takes place (de Lamirande and Gagnon, 1998; Breitbart, 2003).

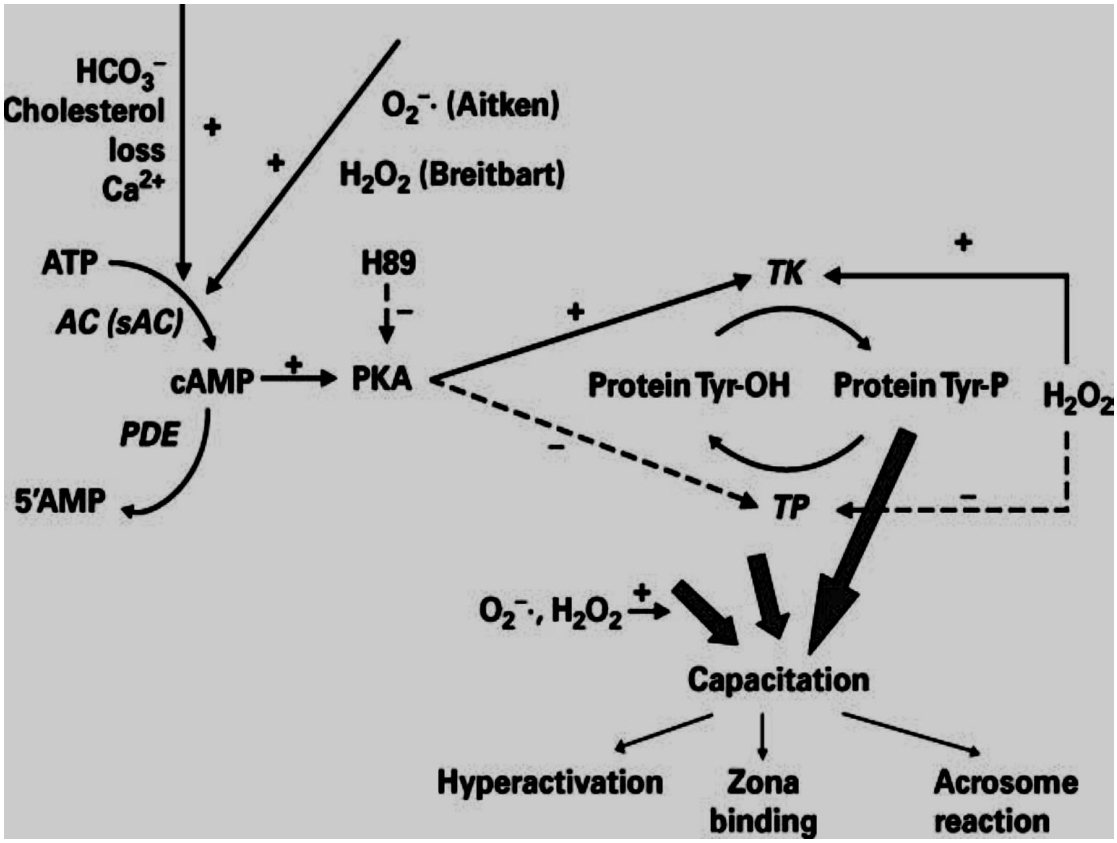


**Figure 2. 2: Effects of ROS on intracellular signalling during sperm capacitation (Adapted from Breitbart, 2003)**

*In vitro* study studies carried out on mouse spermatozoa have shown the increase of fertilization of 50% by induction of mild LPO using a combination of ferrous ion and ascorbic acid (Kodama *et al*., 1996, de Lamirande *et al*., 1997). According to the study, high hydrogen peroxide-induced OS stimulates the activity of sperm function and increases the rate of fertilization. Superoxide anion stimulates the capacitation through the action of an oxidase in incubation conditions (de Lamirande *et al*., 1997). Further stimulation of ROS production; superoxide anion, hydrogen peroxide cause the release of unesterified fatty acid from the plasma membrane of these cells (de Lamirande *et al*., 1997).

### 2.2.2 Reactive oxygen species and male infertility

Reactive oxygen species dysfunction causes a state of OS. This is due to the imbalance between ROS and their scavengers in body system thereby causing harmful effects on cell: damage of DNA, oxidations of polyunsaturated fatty acids in lipids (LPO), oxidations of amino acids in proteins and oxidatively inactivate specific

enzymes by oxidation of co-factors (Agarwal *et al*., 2014). Consequently, OS has been implicated in numerous disease states such as cancer, connective tissue disorders, aging, infection, inflammation, acquired immunodeficiency syndrome, and male infertility (Kullisaar *et al*., 2012).

The high levels of ROS production in the male reproductive tract has become a real concern because of their potential toxic effects on sperm quality and function (Makker *et al*., 2009). When ROS exceed the antioxidant capacity of the seminal plasma it results in OS which is harmful to spermatozoa (Agarwal *et al*., 2014). Oxidative stress may cause infertility by two principal mechanisms: first, OS damages the sperm membrane which in turn reduces sperm motility and ability to fuse with the oocyte. Secondly, OS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo (El-Tohany, 2012). Moreover, high ROS production may accelerate the process of germ cell apoptosis, leading to a decline in sperm count, DNA damage and poor outcome of fertility (Krieger-liszkay, 2004; Agarwal *et al*., 2014) (Figure 2.3).

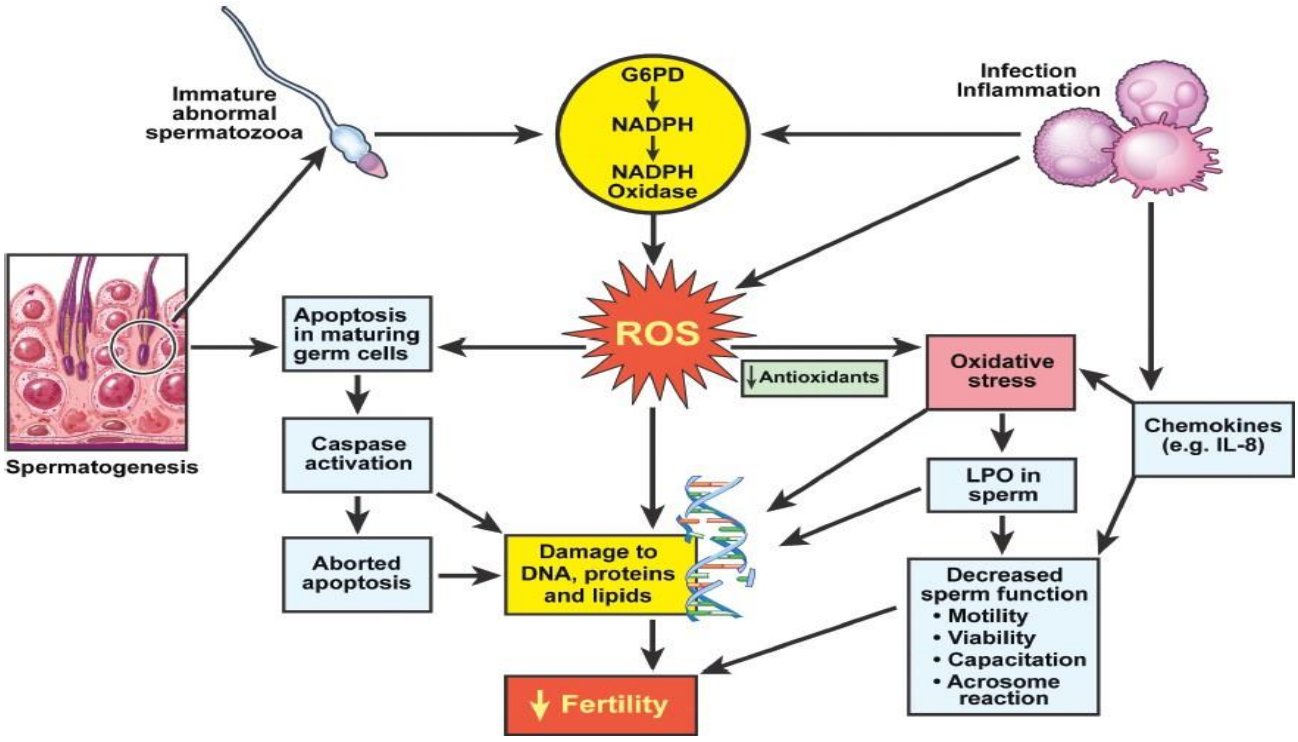


**Figure 2.3: Mechanism of oxidative stress in human semen (Adapted from Agarwal *et al*., 2014)**

### 2.2.2.1 Oxidative stress and sperm membrane lipid peroxidation

In mammals, the epididymis tubule linked to the testis and serves to store sperm produced. During epididymis transit, the sperm reaches $10^{10}$ cells/ ml at the same time as the sperm motility and metabolism increases, with the threat of oxidative stress for gametes (Dacheux, 2003). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective (Huszar *et al.,* 1997, Agarwal *et al*., 2003) with a high content of polyunsaturated fatty acids (PUFAs) rich membrane, plasmalogens and sphingomyelins **(**Aitken *et al*., 2007**;** Aitken and De Iuliis, 2010). The retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation. Reactive oxygen species may bind with biomolecules and change their structure. For instance, major cell damages result from the ROS-induced alteration of polyunsaturated fatty acids in membrane lipids, essential proteins and DNA (Aitken and Roman, 2008).

Lipid peroxidation is the oxidation of polyunsaturated fatty acids that contain more than two carbon double bonds (Maneesh and Jayalekshmi, 2006). The most common types of LPO are nonenzymatic membrane LPO, and enzymatic (NADPH and ADP dependent) LPO. The enzymatic reaction involves NADPHcytochrome P-450 reductase and proceeds via an ADP-$Fe^{3+}$, $O_2^{\cdot -}$ (perferryl) complex and produce an end product of LPO as malondialdehyde (MDA) in spermatozoa (Ernster, 1993; Saraswat *et al*., 2014). Malondialdehyde reacts with the free amino group of proteins, phospholipids and nucleic acids by altering their structure which induces dysfunction of immune systems, mutation and cancer within the cell (Mao *et al*., 1999; Lee *et al*., 2004). The most significant effect of LPO in spermatozoa leads to a loss of integrity and fluidity in the sperm plasma membrane by altering the structural and functional parameters (Henkel, 2005; Agarwal *et al*., 2006). The loss of membrane fluidity causes abnormal ATPases that regulates the intracellular concentrations of nutrients and ions (such as sodium or calcium). The increasing accumulation of ions leads to sperm cell destruction (Ernster, 1993).

### 2.2.2.2 Oxidative stress and DNA damage

It is well known that oxidative damage to mitochondrial DNA occur in all aerobic cells rich in mitochondria and this may include spermatozoa (Badade and Samant, 2011). DNA damage in spermatozoa has been associated with reduced rates of fertilization; increased of miscarriage and morbidity in the offspring (Zini and Sigman, 2009; Aitken and De Iuliis, 2010). Many possible causes of DNA damage exist and include abortive apoptosis, infection, defective spermatogenesis, and OS (Aitken *et al*., 2007) (Figure 2.3). DNA damage is often induced by OS, rather than being the result of other processes such as defective apoptosis (Kodama *et al*., 1997; Barroso *et al*., 2000). Such DNA damage may accelerate the process of germ cell apoptosis, also known as programmed cell death. Apoptosis in the testis can lead to a decline in sperm count and result in infertility (Sun *et al*., 1997). Moreover the structure and function of spermatozoa changes and they become vulnerable to macrophage attack (Aitken *et al*., 1994).

Oxidative stress has also been associated with high frequencies of single and double DNA strand breaks (Twigg *et al*., 1998). Reactive oxygen species induces DNA damage in the form of modification of all bases (primarily guanine via lipid peroxyl or alkoxyl radicals), production of base-free sites, deletions, frameshifts, DNA cross-links through covalent binding to MDA, and chromosomal rearrangements (Duru *et al*., 2000). Reactive oxygen species can also cause gene mutations such as point mutation and polymorphism, resulting in decreased semen quality (Spiropoulos *et al.*, 2002 and Sharma *et al*., 2004).

### 2.2.2.3 Oxidative stress and apoptosis

Apoptosis is a non-inflammatory response to tissue damage characterized by a series of morphological and biochemical changes (Elgazzar, 2014). Cellular apoptosis appears to be a constant feature in the adult testis and during early development (Shaha *et al*., 2010). It helps in the elimination of abnormal spermatozoa by the regulation of extrinsic and intrinsic factors in the presence of different stimuli (Agarwal, 2005).

Mitochondria play a key role in the mechanism of apoptosis (Agarwa, 2005; Renault and Chipuk, 2014). The integrity of mitochondria is established by the presence of cytochrome C in the inner membrane space (Said *et al*., 2004; Renault and Chipuk, 2014; Weaver *et al*., 2014). Two distinct pathways (intrinsic and extrinsic) exist in the process of caspase activation in mammalian cells (Elkholi and Chipuk, 2014). The intrinsic pathway involves the proapoptotic Bcl-2 protein-dependent release of cytochrome c from the mitochondria into the cytosol, where it binds to the apoptotic protease activating factor 1 (Apaf- 1), dATP and procaspase-9 (Shi, 2002; Newmeyer and Ferguson-Miller, 2003; Payá *et al*., 2014). The fusion of this multiprotein complex named apoptosome results in the activation of initiator caspase-9 and the subsequent proteolytic processing and activation of effector caspases3, 6 and 7 (Riedl and Shi, 2004; Borutaite, 2010) while the extrinsic pathway involves the binding of a death receptor such as Type I membrane protein (Fas) to its ligand FasL (Danial and Korsmeyer, 2004; Czabotar *et al*., 2014).

Reactive oxygen species initiates chain reactions activating mitochondria to release cytochrome with caspase activation which induces apoptosis (Said *et al*., 2004; Agarwal *et al*., 2007). Caspases belong to a family of cysteine proteases involved in apoptotic cell death and can be divided into two major groups: initiator caspases and effector caspases (Riedl and Shi, 2004). Once activated, initiator caspases (8, 9 and 10) cleave downstream procaspases to generate an amplification cascade. Effector caspases (3, 6 and 7) degrade many cell substrates like actin, poly ADP-ribose polymerase (PARP) and Golgi apparatus proteins like Golgin-160 or p115, resulting in fragmentation of DNA and apoptotic bodies (Stroh and Schulze-Osthoff, 1998; Hengartner, 2000; Chiu *et al*., 2002).

Therefore, high levels of ROS disrupt the inner and outer mitochondrial membranes, release out cytochrome C protein to activate the caspases and induce apoptosis (Wang *et al*., 2003) (Figure 2.4). Apoptosis in sperm may also be initiated by ROS independent pathways involving the cell surface receptor called Fas or CD 95. Fas is a death receptor which belongs to the tumour necrosis factor (TNF) receptor family. When it joins with Fas ligand (FasL), it gets activated and reacts with an adaptor protein called Fas associated death domain (FADD) protein. This interaction between activated Fas and FADD allows pro-caspase 8 to join the complex Fas–FADD. Pro-caspase 8 is then immediately cleaved to form caspase 8, which in turn

activates caspase 3, triggering apoptosis. Caspase 8 also stimulates another protein called BId to its active form tBId, which inserts in the mitochondrial membrane and stimulates release of cytochrome C, an important apoptotic factor (Schimmer *et al.*, 2001; Saleh *et al*.,2003) (Figure 2.4).

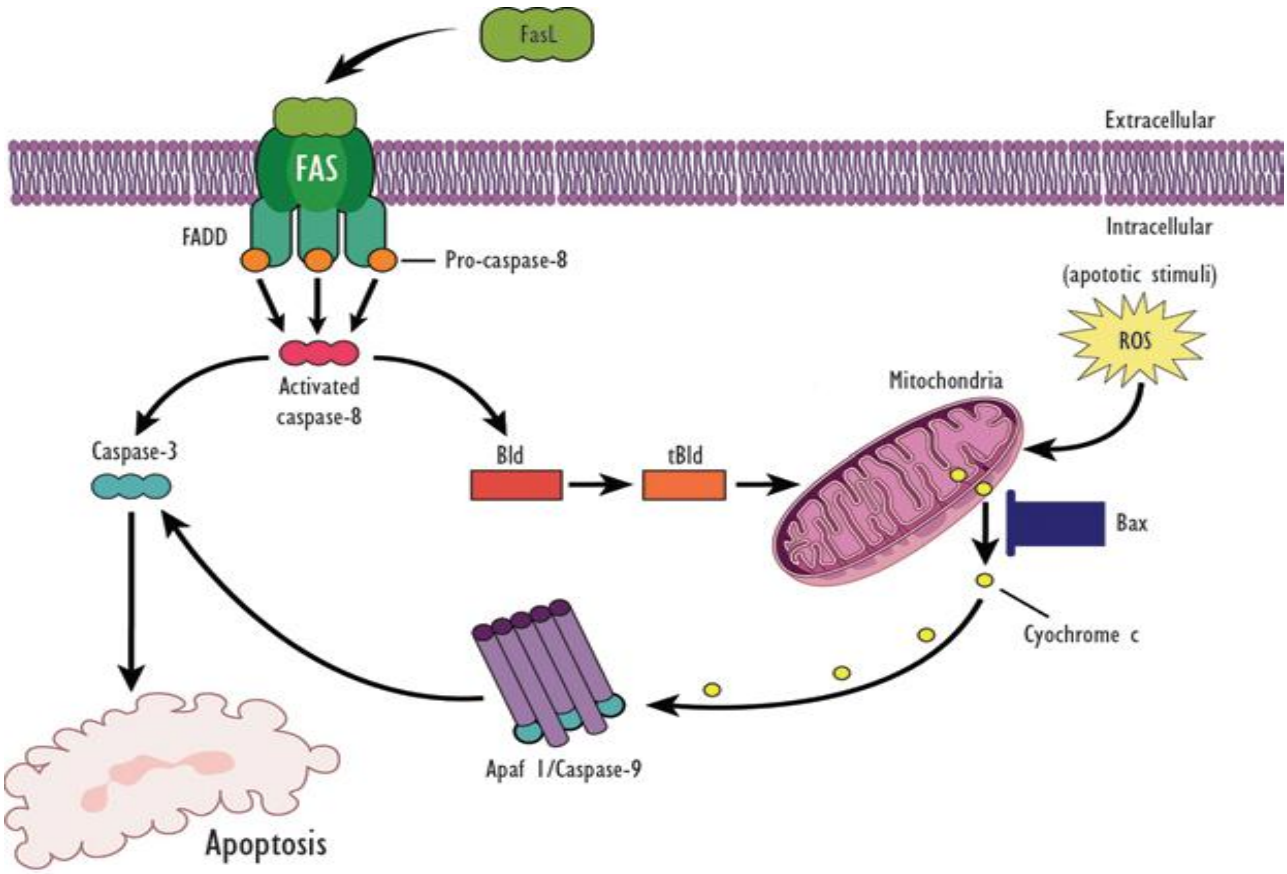


**Figure 2.4: Activation of caspases which induce apoptosis (Adapted from Agarwal, 2005)**

## 2.3 Antioxidants and male reproductive function

An antioxidant is a molecule that inhibits the oxidation of other molecules by controlling or preventing the excess free radicals which are ROS and RNS (Matés, 2001). In general, the antioxidant defence present in reproductive organs aim to protect gonadal cells and mature spermatozoa from OS damage (Sikka, 2001).

Antioxidant defence system mechanisms include three levels of protection: prevention, interception and repair. Antioxidants prevent at the first line to fight

against an oxidative insult. For example, antioxidant enzyme with metal ions, iron and copper ions co-factor bind to the chain reaction of free radicals and prevents its propagation (Sies *et al*., 1993). Second line of defence; the interception has to deactivate the process of free radicals formation and conducts to non-radicals molecules (sies, 1992). During free-radical induced damage, antioxidants repair the damages. However, spermatozoa are unable to repair the damage induced by ROS because they lack the cytoplasmic enzyme systems required to accomplish this (Irshad and Chaudhuri, 2002). The pathological levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma (Lewis *et al*., 1995). Additionally, antioxidants had been used to treat seminal OS thereby improve fecundation and the outcome of assisted reproductive technologies (Agarwal and Sekhon, 2010). In these cases, antioxidants might remove free radical induced infertility and maintain pregnancy.

Oxidative stress plays an important role in the aetiology and progression of major human degenerative diseases which has triggered enormous and worldwide interest in endogenous and exogenous antioxidants (Basu *et al.*, 1999). Endogenous antioxidants include both enzymatic and non-enzymatic antioxidants, while exogenous antioxidants are supplementary substances mostly found in fruit and vegetables (Agarwal, 2005).

### 2.3.1 Endogenous antioxidants

Endogenous antioxidants include enzymatic antioxidants and these are able to neutralize and prevent the oxidation of biological molecules (Agarwal *et al*. 2005). Aerobic cells contain the main endogenous enzymatic defence systems; SOD, glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT). These enzymes work together and convert $O_2^-$ and $H_2O_2$ to less reactive species: $H_2O$ and $O_2$ (Agarwal *et al*., 2006) (Figure 2.5).

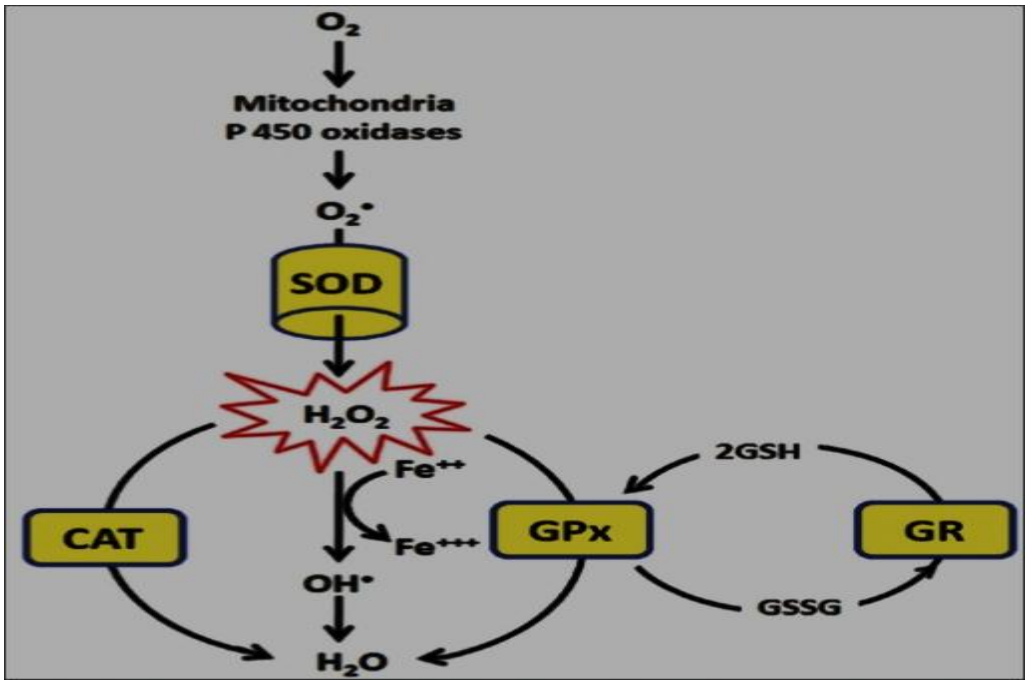


**Figure 2.5: Endogenous enzymatic defence systems of all aerobic cells (Adapted from Agarwal *et al*., 2006)**

### 2.3.1.1 Antioxidant enzyme mechanisms and male reproductive organs

#### i. Superoxide dismutase

Superoxide dismutases (SOD's) are metalloenzymes which catalyse the dismutation reaction of the $O_2^-$ into $H_2O_2$ and $O_2$ (Johnson and Giulivi, 2005) (Figure2.5). In mammals, SOD's are found in different forms and classified into three types depending on the transition metal ion found at their active site and number of subunits (Miller, 2004). Two of the intracellular forms are copper–zinc SOD, which are localized mainly in the cytoplasm and contains copper and zinc (Cu, ZnSOD, SOD–1) in the active centre which is a homodimer of 32 kDa, and manganese SOD, which is located mainly in the mitochondrial matrix with manganese at its active center (MnSOD, SOD–2), an 89 kDa homotetramer. The extracellular form of SOD (EC–SOD, SOD–3) acts in the extracellular space and a tetrameric glycoprotein of 135 kDa (Schreibelt *et al*., 2007).

Superoxide dismutase destroys $O_2^-$ by the successive oxidation and reduction of the transition metal ion at the active site in a "ding dong" mechanism with remarkably

high reaction rates (Meier *et al*., 1998). Every type of SOD binds single charged anions such as azide and fluoride. Distinctly, differences have been noted in the susceptibilities of $Fe^{-}$, $Mn^{-}$ or Cu/Zn-SODs. Cu/Zn-SOD is competitively inhibited by azide ($N3^{-}$), cyanide ($CN^{-}$) and fluoride ($F^{-}$) (Leone *et al*., 1998; Vance and Miller, 1998). Superoxide dismutase must be conjugated with CAT or GPx to prevent LPO of the plasma membrane (Junichi *et al*., 2005). Superoxide dismutase also prevents premature hyperactivation and capacitation induced by superoxide radicals before ejaculation (Lamirande *et al*., 1997). An abnormality in SOD production can result in an uninterrupted generation of ROS and damage to both testicular tissue and spermatozoa (Maiorino and Ursini, 2002).

### ii. Catalase

Catalase is an intracellular antioxidant enzyme that is mainly located in cellular peroxisomes and to some extent in the cytosol of mammalian cells. Catalase is a tetrameric enzyme consisting of four identical, tetrahedrally arranged subunits of 60 kDa each containing a haem group and NADPH in its active centre (Schreibelt *et al*., 2007). Consequently it exerts two types of activities, namely catalytic and peroxidic. Catalase favours the decomposition of $H_2O_2$ into $H_2O$ and $O_2$ (Figure 2.5). Thus removes free radical chain reaction are responsible for LPO (Agarwal *et al*., 2008). It removes $O_2^{-}$ generated by NADPH-oxidase in neutrophils and can play an important role in protecting spermatozoa during genitourinary inflammation (Baker *et al*, 1996). It is also improves motility and protects spermatozoa to survive within the female tract against $H_2O_2$ (Agarwal, 2005).

### iii. Glutathione peroxidases

Glutathione peroxidase was first discovered in 1957 when it was found that it protects erythrocytes against oxidative damage (Mills, 1957). In addition, GPx has been reported as a group of selenoproteins composed of tetramers of four identical subunits (monomer 22-23KDa) (Herbette *et al.*, 2007 and Chabory *et al*, 2010). This enzyme requires selenium as a cofactor and contains a selenocysteine amino acid residue in the active site of each monomer (Margis *et al.,* 2008). Quite extensively studied in eukaryotes, the mammalian GPx family is divided into 8 classes (GPx1 to

GPx8) based on GPx proteins and sequences (Brigelius-Flohé and Maiorino, 2013). These classes participate in different functions in organisms.

Among the different antioxidant enzymes encountered in eukaryotic cells, GPx occupies a critical position in the cascade of ROS-recycling events, allowing for small physiological adjustments in the concentrations of $H_2O_2$ or other substrates, such as organic peroxides. Glutathione peroxidase specifically plays an important role in the detoxification of peroxides in the cell. It prevents destruction of cell membranes since peroxides decompose in high reactive free radicals and therefore helps in preventing LPO of cell membranes (Szasz *et al.,* 2007; Covarrubias *et al.,* 2008). Glutathione peroxidase removes peroxyl radicals from various peroxides including $H_2O_2$, improves sperm motility, is essential for embryogenesis and has an effect on male fertility (Pfeifer *et al*., 2001; Maiorino *et al*., 2003; Moreno *et al*., 2003).

### 2.3.2 Exogenous antioxidants

The human body naturally produces antioxidants, but the process is not 100 percent effective when there is an overwhelming production of free radicals, while the efficiency also declines with age (Sen *et al*., 2010). The body needs exogenous dietary antioxidants, either natural or synthetic to perform optimally (Bouayed and Bohn, 2010). Exogenous antioxidant supplementation can be achieved through the dietary intake of natural vitamins and minerals found in animal products, fruits and vegetables, vegetable oils and natural beverages (Moure *et al*., 2001; Van Langendonckt *et al*, 2002). Moreover it can also be achieved through diatary intake of synthetic food additives (Bouayed and Bohn, 2010).

Supplementary antioxidant intake can support endogenous antioxidants to prevent and lower the risk of diseases (Carlsen, 2010). They protect the organism more efficiently against OS which cause the development of diseases by interfering with oxidative damage to the DNA, proteins, and lipids (Rosa*,* 2010). Moreover, they prevent and inhibit the propagation of ROS production (Hu and Kitts, 2000; Nicolle *et al.,* 2003*)*.

### 2.3.2.1 Antioxidant vitamins

Antioxidant vitamins are mostly derived from plant material such as fruits, seeds and leaves (Moure *et al*., 2001; Van Langendonckt *et al*, 2002). They are main types of vitamins and most are widely used in dietary supplements to prevent health disorders due to OS (Mangge *et al*., 2014). Ross *et al* (2010) assumed that the low cost and relatively low risk of toxicity of vitamins are mostly used in clinical trials especially to treat OS- induced male factor infertility. They can be used in different manners such single treatment (using one vitamin) or in combination (using two vitamins) (Baker *et al*., 1996) several mechanisms that include exogenous nutrients and endogenous antioxidant systems have evolved to protect us (Kahl, 1991) from inevitable intracellular or extracellular oxidative stress. The most widely known antioxidant nutrients are derived from phytochemicals (plant-derived chemicals) and include polyphenols (e.g. flavonoids, anthocyanidins, catechins or stilbenes). Multiple antioxidant properties of flavanoids can be seen and each with different mechanisms of action. In particular, they trap reactive oxygen species (ROS) and inhibit enzymes involved in the production of oxidative stress. Flavanoids also block radical formation through Fenton-type reactions and can regenerate other antioxidants, such as tocopherol (vitamin E) (Rice-Evans, 2001).

Vitamin E is an important hydrophobic antioxidant and exists in eight forms such as α-, β-, γ- and δ-tocotrienols and α-, β-, γ- and δ-tocopherols (Langseth, 1995; Hensley *et al*., 2004). It has to protect sperm membrane against LPO by using two different mechanisms; firstly, it directly scavenges ROS. Secondly, this vitamin up-regulates antioxidant enzymes such as SOD, GPX, GRD, CAT and NADPH (Bolle *et al.*, 2002; Vertuani *et al*., 2004; Makker *et al.,* 2009). Conventionally, this effect is due to α-tocopherol and ascorbic acid function together to inhibit the process of LPO (Pryor, 2000). Kojo (2004) has described the antioxidant mechanism reaction of α-tocopherol; thus is converted to a α-tocopherol radical by the donation of labile hydrogen to a lipid or lipid peroxyl radical, and the α-tocopherol radical can therefore be reduced to the original α-tocopherol form by ascorbic acid.

The protective effects of vitamin E have been observed *in vitro* studies and *in vivo*. Vitamin E supplementation appears to have a dose-dependent effect and neutralises

free radicals by protecting sperm motility and morphology. The supplementation of vitamin E (10mmol/l) reduced LPO and improved sperm motility. Additionally, vitamin E(10mmol/l) has been added to cryoprotectants during cryopreservation and thawing procedures to protect more efficiently OS and loose of sperm motility generated from these procedures( Askari *et al*., 1994). Another example, administration of 100 mg of Vitamin E three times a day for 6 months in a group of asthenozoospermic patients with normal female partners showed a significant decrease in sperm lipid peroxidation and increased motility and pregnancy rates (Kessopoulou *et al*., 1995; Keskes–Ammar *et al*., 2003). Moreover vitamin E may be combined with vitamin C to protect spermatozoa against ROS attack (Baker *et al*., 1996).

Vitamin C (ascorbic acid) is a potent hydrophilic antioxidant in extracellular fluids and has the ability to remove of hydrogen peroxide radicals and prevents sperm agglutination (Agarwal *et al*., 2007). This high antioxidant potency has been shown in protection of sperm membrane against LPO (Chan *et al*, 1993) and sperm DNA from the harmful effects of ROS (Fraga *et al*., 1991). Genetic disorders in smoking polulation have been treated with vitamin C supplementation, thus may minimise endogenous oxidative DNA damage (Agarwal and Sekhon, 2010).

Vitamin C and vitamin E act synergistically by recycling α-tocopherol from α-tocopherol radicals in membranes and lipoproteins (Carr and Frei 1999; Kojo 2004). It is also increases intracellular glutathione levels in protection against free radicals attack cell membrane (Naziroglu and Butterworth 2005). For this, administration of 200 mg of vitamin C orally along with vitamin E and glutathione for 2 months significantly reduced hydroxyl glutathione levels in spermatozoa and also led to an increase in sperm count (Agarwal *et al*., 2008). Although, some investigation has established that vitamin C was more effective than vitamin E in reversing ROS-induced mouse embryo toxicity and improve the blastocyst development rate (Wang *et al*., 2002).

#### 2.3.2.2 Phytochemicals antioxidants

Flavonoids are phytonutrients widely found in fruits and vegetables (Rice-Evans, 2001). They are intake in the form of glycosides and polymers (Hammerstone *et al*

2000, Santos-Buelga and Scalbert, 2000). Flavonoids belong to polyphenolic compounds composed of three benzene rings with hydroxyl (OH) groups (Romero *et al*., 2013). Depending on the degree of hydroxylation and the presence of double bonds in the heterocycling pyrone ring, flavonoids can be divided into thirteen classes and the most important are the flavonols, flavanols, flavones, isoflavones, anthocyanidins or anthocyanins and flavanones (Scalbert and Williamson2000, Moreno, 2002; Romero *et al*., 2013) (Figure 2.6).

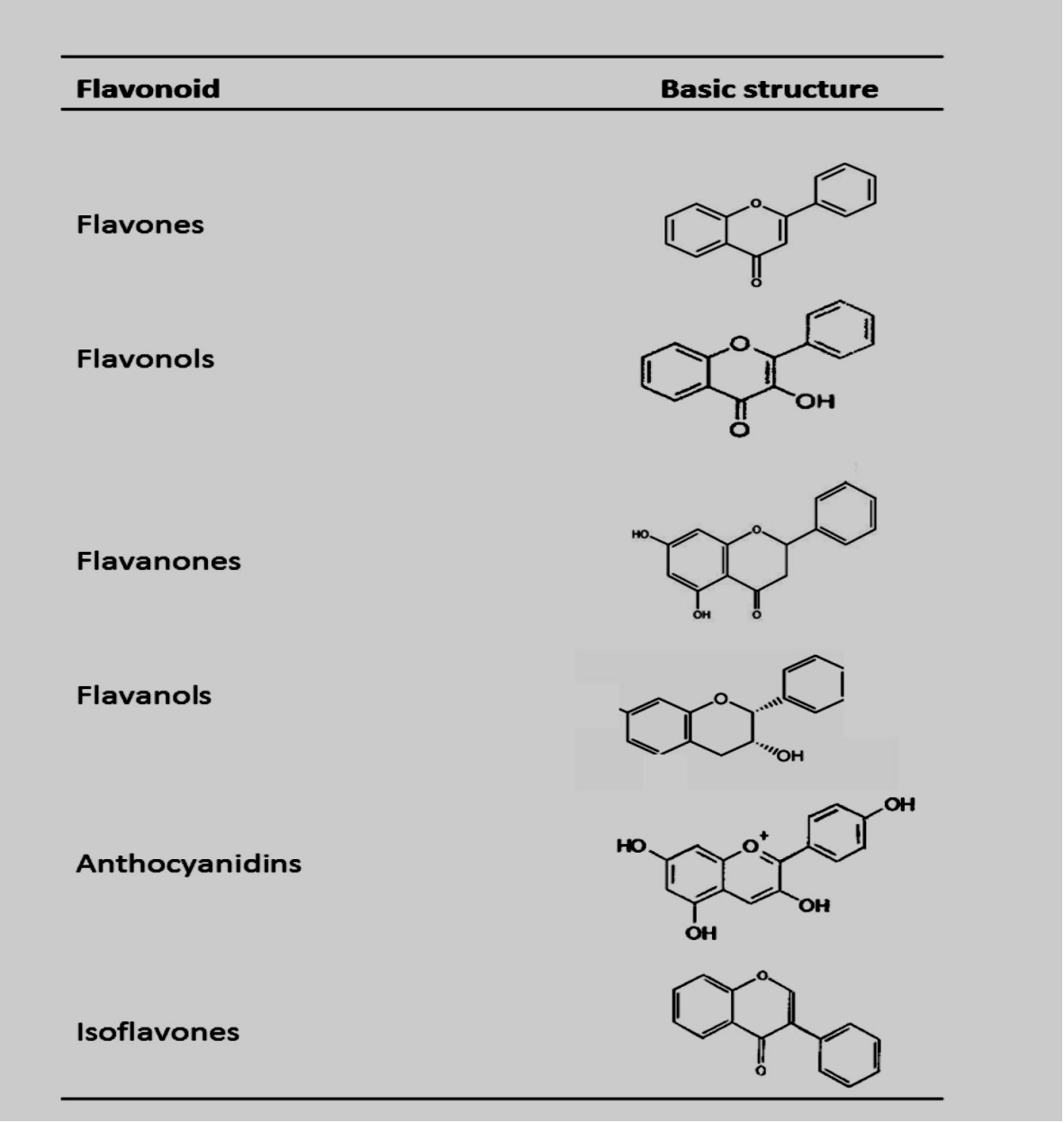


**Figure 2.6: Basic chemical structures of the main classes of flavonoids (Adapted from Romero *et al*., 2013)**

The flavonoids retain pharmacological activity due to their low toxicity, presenting in general activity on the vascular system with vitamins action where flavanoids protect the vascular wall by decreasing permeability and increasing of capillary resistance (Mulvihil, 2010; Retta, 2012). Therefore flavonoids work synergistically with other vitamins due to the ascorbic acid which reduces the oxidation (Romero *et al.,* 2013). Similarly, they possess biological properties depending on the nature, position of the substituents and the number of hydroxyl groups (Schroeter *et al*., 2002). These properties are anti-viral, anti-allergic, antiplatelet, anti-inflammatory, anti-tumor and antioxidative (Schroeter *et al*., 2002). The antioxidant activity of flavonoids increases because of its structure; hydroxyl group on B-ring, presence of unsaturation and of 4-oxo function in the C-ring (Farkas *et al*., 2004).

Mechanisms of antioxidant action can include suppression of ROS formation either by inhibition of NADPH oxidases or by chelating trace elements and upregulation or protection of antioxidant defences (Mishra *et al.,* 2013) (Figure 2.7) . Flavonoids act indirectly to inhibit oxidative process by helping some antioxidant enzymes; CAT and SOD to neutralize ROS (Schroeter *et al.,* 2002). It has been found flavonoids prevent lipid peroxides (Schroeter *et al.,* 2002). Moreover, flavanoids have been observed to protect against neurogenerative diseases by preventing OS (Rice-Evans, 2001; Christopher, 2009). For example, flavonoids have been shown to retain antihyperglycaemic activity in diabetes related induced OS in animal models (Rahman, 2003; Rahimi *et al*., 2005).

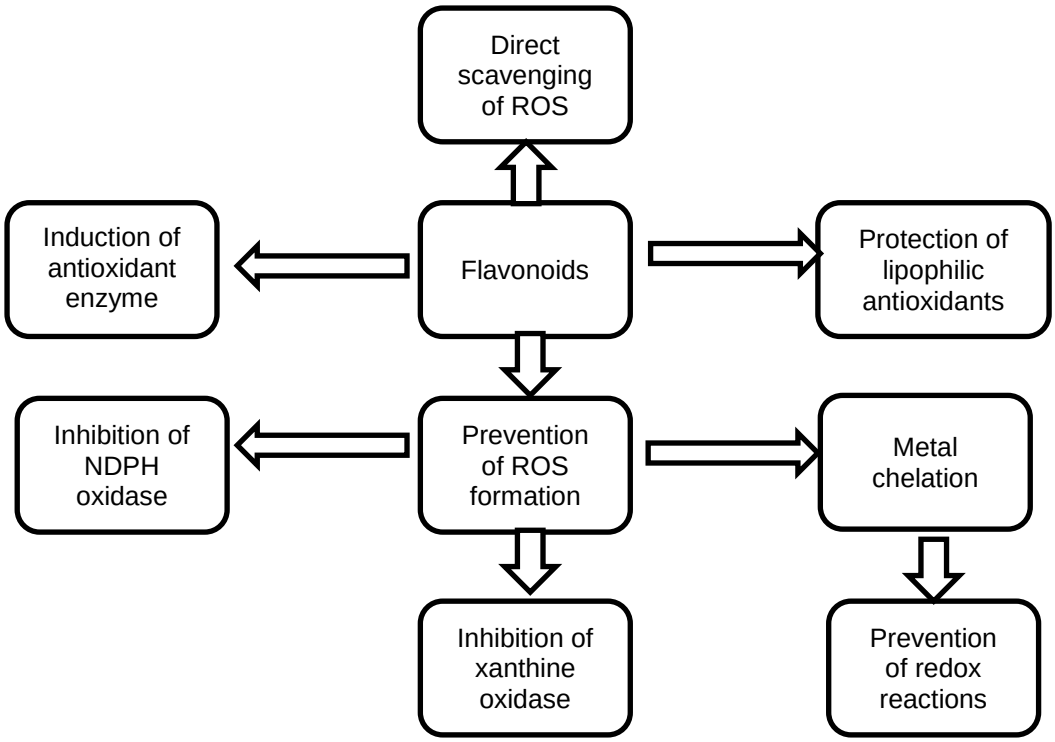


**Figure 2.7 Mechanism of antioxidant effects of flavonoids (Adapted from Mishra *et al*., 2013)**

Dietary flavonoids have chemopreventive and estrogenic properties and these may protect male reproductive organs function (Galati and Brien, 2004). Flavonoids may play role a role in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen-detoxifying systems (Galati *et al.,* 2000; Birt *et al.,* 2001; Ren *et al*., 2003). Flavonoids as phenolic compound are phytoestrogens with nonsteroidal properties, but with oestrogen-like biological activity (Galati and Brien, 2004). Oestrogen is found in both males and females but not only in their reproductive systems as it is a hormone present in the blood (Pelletier *et al*., 2000). It furthermore has several other functions apart from reabsorbing epididymal fluid. Disruption of this essential function causes the decreased of sperm concentration which has been observed in infertility (Hess *et al*., 1997).

## 2.4 Diabetes mellitus related oxidative stress and male infertility

Diabetes mellitus is a complex group of diseases characterized by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action or both (Gavin *et al*., 1997). It is classified into type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2 DM). Type 1 diabetes mellitus develops when the insulin producing β-cells have been destroyed and are unable to produce insulin. Type 2 diabetes refers to insulin resistance when the body is unable to produce an adequate amount of insulin or the insulin which is provided does not work powerfully (Singh *et al.,* 2011). Both types of diabetes are associated with OS. Oxidative stresses in diabetic patients develop from pathways which include the non-enzymatic, enzymatic and mitochondrial signalling pathways (Ahmed *et al.*, 2005; Arulselvan *et al*., 2012) (Figure 2.8).

Hyperglycaemia results in an increase in ROS levels. The glucose undergoes autoxidation and glucose also reacts with proteins leading to the development of amadori products followed by formation of advanced glycosylation end products (AGE's). In hyperglycaemia, there is enhanced metabolism of glucose through the polyol (sorbitol) pathway, which also results in enhanced production of superoxide (Ahmed *et al.*, 2005)**.** Another enzymatic generation of ROS is via the mitochondrial respiratory chain through the oxidative phosphorylative process where electrons are transferred from electron carriers NADH and $FADH_2$, through four complexes in the inner mitochondrial membrane, to oxygen, generating ATP in the process (Green *et al*., 2004). Hyperglycaemic conditions disturb endothelial cells and ROS are produced which participate in the development of diabetic complications. Enzymatic over-productions of ROS in diabetes through NADPH oxidase enhance $O_2^{-\cdot}$ (Guzik *et al*., 2002). The above-mentioned sources with different pathways lead to OS in diabetes which results in diabetic complications such as macro and micro-vascular dysfunctions (Duckworth, 2001; Ahmed *et al.*, 2005).

Diabetes-related to OS, endocrine disorders and neuropathy may contribute to reproductive impairment by causing sexual function alterations including testicular function and epididymal sperm transit (Amaral *et al*., 2008). Oxidative stress result from autoimmune damage of T1DM produces defective sperm nucleus, DNA

damage and germ cell apoptosis (La Vignera *et al*., 2012). Retrograde ejaculation, a condition where the semen passes backward into the bladder during ejaculation, has also been reported in some diabetic men who suffer from autonomic neuropathy (Vinik, 2003).

Muralidhara (2007) reported that reproductive organ functions in STZ-induced diabetic animals were impaired, causing the decline of fertility. The diabetogenic agent STZ is a monofunctional nitrosourea derivative that has a broad-spectrum antibiotic activity. When STZ enters the β-cells through the GLUT2 glucose transporters, these cells become necrotic. The methylation potential of STZ breaks DNA thereby producing inactive chromosomes. Subsequently, these cells die and cannot produce insulin which modulate for T1DM creation (Bolzan *et al*., 2002; Muralidhara, 2007, Ayepola *et al*., 2014).

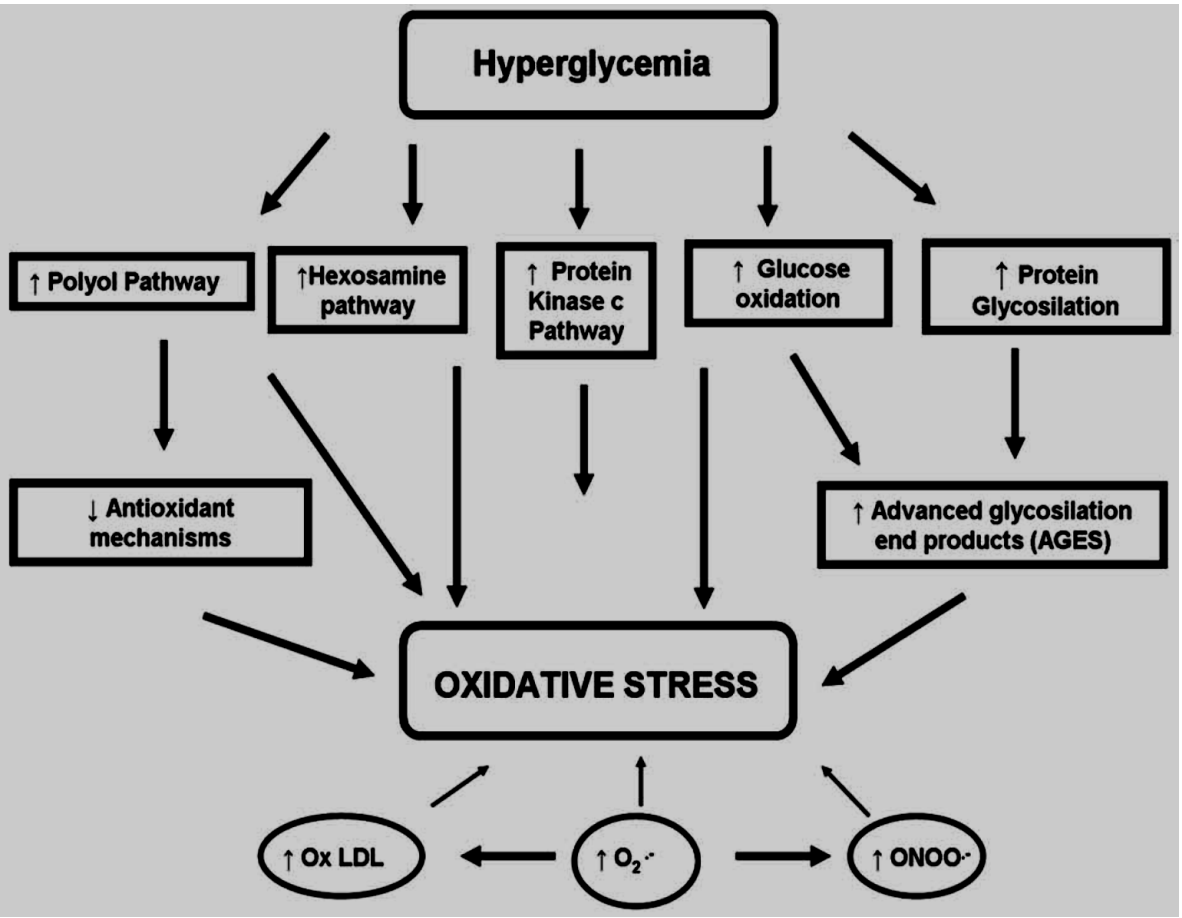


**Figure 2. 7: Hyperglycaemia-induced oxidative stress (Adapted from Ahmed *et al.*, 2005)**

### 2.4.1 Role of insulin in male reproduction

In mammals, reproduction is controlled by the hypothalamic-pituitary-gonadal (HPG) axis, but it is also modulated by changes in energy homeostasis and metabolism (Burks *et al*., 2000). In a normally functioning HPG axis; the hypothalamus releases gonadotropin-releasing hormone (GnRH) in a pulsatile fashion. This stimulates the basophilic cells of the pituitary to secrete both luteinizing hormone (LH) and follicle stimulating hormone (FSH). Luteinizing hormone and FSH act on Sertoli cells and Leydig cells respectively, to stimulate and regulate the process of spermatogenesis (Schoeller *et al.*, 2012) (Figure 2.9).

Insulin is a natural endocrine secretion which maintains homeostasis by regulating blood glucose levels by acting on the cellular uptake of glucose (Obici *et al*., 2002). The effects of insulin in the function of the male reproductive tract might be mediated through insulin receptors and leptin hormone in communication with the HPG axis or through the direct interaction of insulin on the testes and spermatozoa cells (Boura-Halfon and Zick 2009).

The cascade of insulin to produce energy in the brain via insulin receptors substrate (IRS-2) through phosphatidyl inositol 3-kinase (PI3-kinase) and activates mediator signalling of energy; protein kinase (Boura-Halfon and Zick 2009). The brain gains energy and releases it to the whole body to maintain homeostasis (Porte *et al*., 2005). The insulin family of growth factors (insulin, insulinlike growth factors I (IGF1) and II (IGF2) are small single-chain mitogenic polypeptides that provide essential signals for the control of growth, metabolism and reproductive functions (Kanzaki *et al*., 2001; Kanzaki and Pessin 2001; Boura-Halfon *et al*., 2009). The insulin/IGF system plays a pivotal role in the regulation of cell growth, proliferation, differentiation and survival and affects nearly every organ and system in the body (Efstratiadis, 1998; Nakae *et al*., 2001). Importantly, it also plays a major role in the proper development and function of the testis. Although reproductive capability is regulated by the hypothalamicpituitary-gonadal axis (Roth *et al*., 2011), the activity of local gonadal factors, such as those of the insulin/IGF family, modulate reproductive performance. For example, IGF1 null males are infertile dwarfs and exhibit a reduction of greater than 80% in both spermatogenesis and serum testosterone

levels (Baker *et al*., 1996). This not only highlights the overall importance of IGF signaling for body growth and development, but also emphasizes the critical role of IGFs in reproductive function. The role of some of the receptors, ligands, and signaling molecules of the insulin/IGF signaling pathway and have provided more detailed information that underscores its indispensable role in various aspects of testicular development and function (Nef *et al*., 2003; Pitetti *et al*., 2013). However, the precise roles of IGFs in various facets of testicular development, spermatogenesis and steroidogenesis are still unknown. Insulin and IGF1 activate their tyrosine kinase receptors in the plasma membrane (Kitamura *et al*., 1999), which leads to tyrosine phosphorylation of numerous subtracting proteins including insulin receptor substrate (IRS) proteins (White, 1997).

Leptin, a crucial adipose derived hormone secreted by adipocytes, sends signals to the hypothalamus and also helps to regulate the reproductive system (Schoeller *et al*., 2012). It serves as a metabolic signal that informs the brain of nutritional status and provides information regarding an animal's ability to meet the energy (Barash *et al*., 1996). Leptin receptors are expressed in the testes, suggesting that leptin secreted by fat cells can directly affect the gonads (Zamorano *et al*., 1997). The expression of leptin receptors in the testes seems to be stage-specific, indicating a role in spermatogenesis. Mature spermatozoa suppress leptin receptor expression through a negative feedback loop that prevents the expression of the receptor during the other spermatogenic stages (El-Hefnawy *et al*., 2000). Moreover, leptin and insulin are known to interact with the hypothalamus, ultimately to regulate the output of GnRH from the hypothalamus and thereby controlling of the male reproductive system (Figure 2.9). Both insulin and leptin might also act directly in the testes to regulate spermatogenesis (Roser *et al*., 2001; Fisher *et al*., 2003; Carpino *et al*., 2010; Walters *et al*., 2012). The testes and sperm cells have also been shown to produce protein and contain insulin (Aquila *et al.,* 2005). These cells are activated by insulin to induce serine/threonine protein kinase phosphorylation (pAkt), suggesting a functional role in insulin signalling. Additionally, these cells have been shown to secrete insulin in response to glucose management and spermatozoa release insulin upon capacitation, the process by which sperm acquire the ability to fertilize. This provides a potential mechanism by which insulin has an autocrine role in sperm cells, which could serve to mediate sperm maturation and/or fertilization events

(Schoeller *et al*., 2012). However, abnormal function or the lack of insulin and leptin due to the diseases including DM has an effect on energy production by the cells which impairs reproductive function.

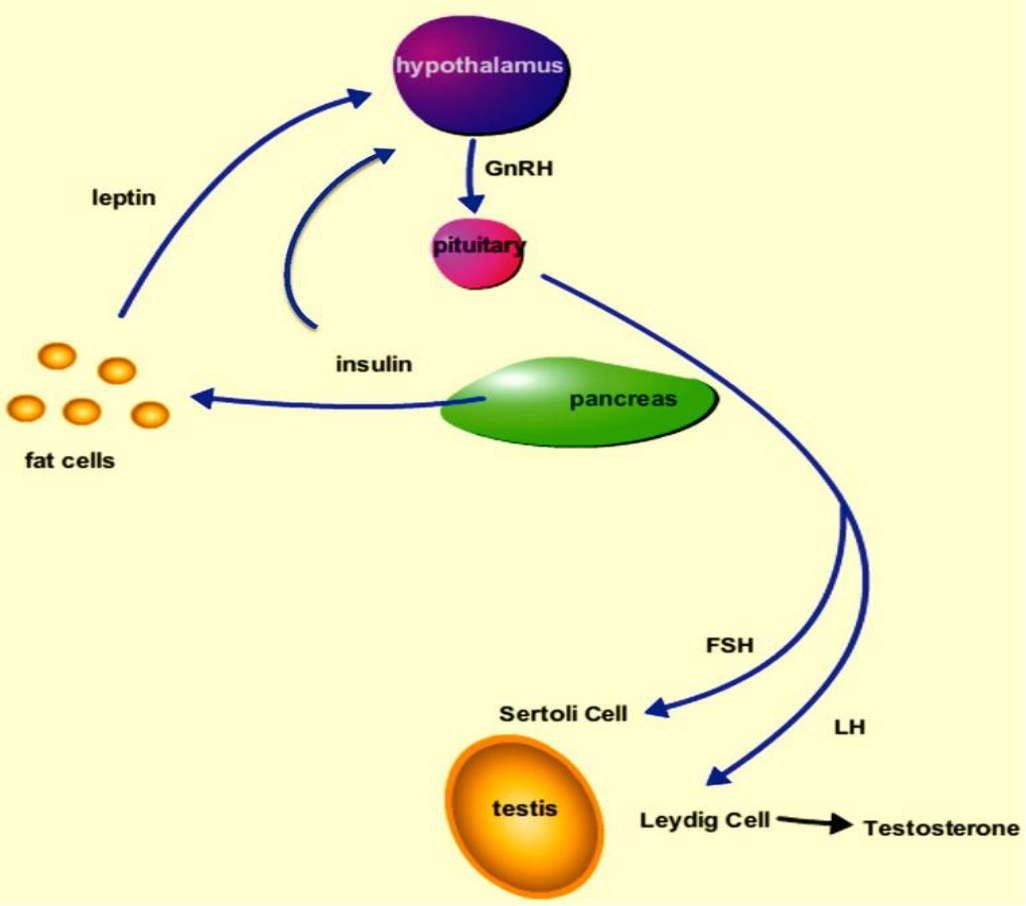


**Figure 2. 8: Representation showing involvement of leptin and insulin in HPG-testis axis (Adapted from Schoeller *et al*, .2012)**

### 2.4.2 Management of diabetes mellitus

Diabetes mellitus is a syndrome implying that efforts targeted at its management should be multifaceted. Adequate consideration should be given to all the accompanying comorbidities and all symptomatic and asymptomatic features. Efforts should be geared towards the attainment of normal or near normal glucose levels. According to Alwan (1994) diabetes management include (i) relieve symptoms (ii) correct associated health problems and reduce morbidity, mortality and economic costs of diabetes (iii) prevent as much as possible acute and long-term complications

(iv) monitor the development of such complications and provide timely intervention and, (v) improve the quality of life and productivity of the individual with diabetes (Alwan, 1994). The orthodox approach to the management of diabetes mellitus has always included lifestyle modification and dietary therapy, administration of oral antidiabetic drugs, and insulin therapy.

Uncontrolled DM can also have a major impact on sexual function, pregnancy related complications, as well as morbidity and mortality of the offspring (Kitzmiller *et al*., 1996). Blood sugar levels can be controlled using a range of various treatment options. Therapeutic options for diabetes include anti-diabetic drugs and a general change in life style habits (Grijesh *et al*., 2009).

#### 2.4.2.1 Pharmaceutical management of diabetes

Pharmacological therapy refers to the use of synthetic antidiabetic drugs to control blood sugar levels in people with DM. Treatment options include insulin, Biguanides; Metformin, Thiazolidinediones, Insulin Secretagogues, Sulfonylureas, Glinides, Alpha Glucosidase Inhibitors, incretins, pramlintide, and bromocriptine (Nathan *et al*., 2006).

Scottish Intercollegiate Guidelines Network (SIGN) has established the guideline for DM management (SIGN, 2010). According to that guidline the management of DM depends on symptoms and type of DM. Generally, individuals diagnosed with T1DM rely on insulin delivered by injection or an insulin pump to help manage their disease. In type 2 diabetes, more intensive treatment strategies have likewise been demonstrated to reduce microvascular complications (Ohkubo *et al*., 1995; UKPDS, 1998). The development of new classes of blood glucose–lowering medications to supplement the older therapies, such as lifestyle-directed interventions, insulin, sulfonylureas, and metformin, has increased the number of treatment options available for type 2 diabetes. Whether used alone or in combination with other blood glucose–lowering interventions, the increased number of choices available to practitioners and patients has heightened uncertainty regarding the most appropriate means of treating this widespread disease (Nathan, 2007)

Statistical averages from the United States indicate that 57.9% of diabetic patients have one or more diabetes-related complications and 14.3% have 3 or more (Mika, 2007). Furthermore, the treatment of diabetes with antidiabetic drugs has been reported to cause secondary effects in diabetic patients (Hermann, 1972; Bailey, 1992; Bastaki, 2005; Kavishankar *et al*., 2011) (Table 2.1) Additionally, DM is a disease with the largest burden on health and thereby contributing the tremendously to economic problems. It is therefore imperative to invest more effort in monitoring of symptoms and treating of long-term complications (Alwan, 1994). Such alternative drugs are necessary because synthetic anti-diabetic drugs are very expensive and may have deleterious side effects on other organ systems. Alternative therapies from natural products like phytomedicines combined with lifestyle changes are needed to improve the treatment of diabetes.

**Table2. 2. Synthetic drugs, mechanisms, site of action, advantages and their side effects (Adapted from Kavishankar *et al.,* 2011)**

| **Antidiabetic drugs** | **Mechanism** | **Site of action** | **Advantages** | **Side effects** |
|---|---|---|---|---|
| Insulin | Controls uptake of blood glucose | Skeletal muscles and fats tissues | Effective antihyperglycaemic | Headache and weight gain |
| Metformin | Decreases insulin resistance | Liver | Weight loss, not hypoglycaemic | Dehydration, gastro intestinal disturbances and respiratory acidosis |
| α-glucosidase inhibitors | Reduces intestinal glucose absorption | Fat, muscle | Decreases postprandial plasma triglycerides | Diarrhoea, abdominal pain |
| Sulphonylureas | Stimulating insulin production by inhibiting the KATP channel | Pancreatic beta cells | Effective and inexpensive | Hypoglycaemia and weight gain. |
| Thiazolidinediones, | Reduce insulin resistance by activating PPAR-γ | GI tract | Low risk | Increased liver enzymes, weight gain, oedema, mild anaemia |

### 2.4.2.2 Phytomedical management of diabetes mellitus.

Ethnobotanical research and literature reviews reported that various plant materials possess antidiabetic activity and have been used for many years in the treatment of diabetes (Rodrigues *et al*., 2012). More than 1200 flowering plants have been claimed to possess antidiabetic properties (Abdel-Hassan *et al.*, 2000; Chang *et al*., 2013; Hilmi *et al*., 2014). This property has been found to be present in different parts of plants such as the aerial parts, bark, flowers, roots, seeds, leaves, bulbs, tubers and/or in the whole plant (Maroo *et al*., 2002). In addition, most medicinal plants may have a definite physiological action on the human body. The bioactive substances include phenolic compounds, tannins, alkaloids, carbohydrates, terpenoids, steroids and flavonoids (Modak, 2007). These aforementioned compounds possess the properties of natural antioxidants as ingested by humans and are able to scavenge free radicals or ROS (Oluwaseun *et al*., 2008).

Some plants, including Ginger roots (Hafez, 2010), *Panax schinseng*, *Trigonella foenum-graecum* (Lucas, 1978) and *Epimedium brevicornum maxim* (Lu, 1994; Noor *et al*., 2000) have been used for treating diabetes and to increase male fertility by using different treatment doses. Studies on animal models showed that these plants have antioxidant (Sekiwa *et al.*, 2000), androgenic (Kamtchouing *et al.*, 2002) and hypoglycemic (AL-Amin *et al*., 2006) activities. The mechanisms of these plants have been shown to inhibit OS and alleviate cytokine-induced impairment. They also stimulate nuclear factor for kappa-light chain-enhancer of activated B cells(NF-κB) to activate and increase intracellular $Ca^{2+}$ which are the most important pathways of insulin production (Rchid *et al*., 2004). Furthermore, these herbal products can increase insulin secretion by increasing size of β-cells in the Islets of Langerhans responsible for producing insulin (Hasani-Ranjbar, 2008).

## 2.5 *Garcinia kola* as phytomedicine plant in human health

Derivatives of plant material have contributed to human health and well-being for centuries. They play a role in development of new drugs which have been traditionally used centuries ago. Approximately 75-80% of the global population is in

support of herbal medicine mainly in developing countries; because of cultural acceptance and less side effects (Spainhour, 2005).

*Garcinia kola* belongs to the Guttiferrae (Clusiaceae) family and grown as a medium sized tree, up to 14 metres high and is highly valued in Nigeria and Central West-Africa where for its use as a medicinal plant and source of food(Okwu, 2005; Geeta *et al*., 2006; Farombi *et al*., 2007).

The fruit, seeds, nuts and bark have been used in African ethnomedicine or traditional medicines for many years as prophylaxis and to cure diseases (Abarikwu *et al*., 2012). The *Garcinia kola* seeds are used both for traditional and medical purposes (Daramola and adegoke, 2011). It has been reported that the chewing of these seeds before meals improved digestion (Adedeji *et al*., 2006). It can also serve as a poison antidote, a tonic and an aphrodisiac (Farombi and Owoeye, 2011). Moreover, *G kola* contains a lot of valuable ingredients useful to humans and animals (Adedeji *et al*., 2008). The phytochemical composition also shows that *G kola* indicates that it can be useful in the pharmaceutical and medical sciences in the formulation of vaccines and supplements that can prevent diseases (Adesuyi *et al*., 2012). An important constituent of the *G kola* seed is biflavonoid (KV) which has clinical properties because of its biological and chemical composition (Braide, 1991; Terashima *et al*., 2002).

### 2.5.1 Kolaviron and chemical composition as source of antioxidants

Kolaviron is an ethanol extract made from G kola seeds (Adegbehingbe, 2008) (Figure 2.10). It is used in both developing and developed countries by traditional healers to treat diseases (Mythilypriya *et al*., 2007). The chemical composition of the bioflavonoids extracted from the seed of G kola was found in a 2:2:1 ratio of bioflavonoid GB1, GB2 and kola flavanone (Iwu *et al*, 1990) (Figure 2.11).

**Figure 2. 9: *Garcinia kola* seeds**
**(Adapted from Adegbehingbe, 2008)**

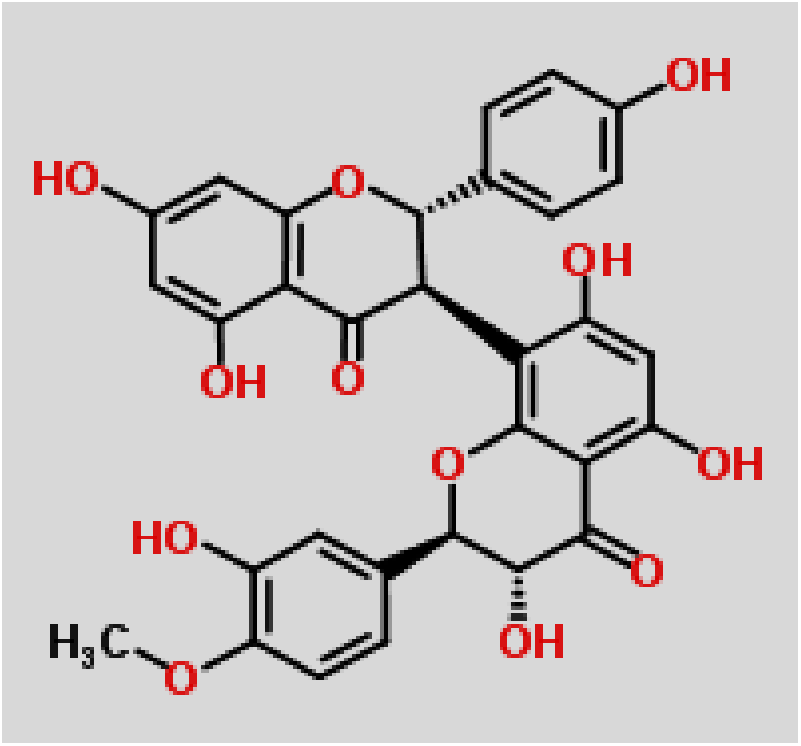


**Figure 2. 10: Chemical structure of kolaviron isolated from Garcinia kola**
**(Reproduced from Iwu *et al*, 1990)**

Kolaviron has been reported to possess potential health effects via biochemical mechanisms including anti-inflammatory, antioxidant, antidiabetic, antiviral, antigenotoxic and hepatoprotective activities (Farombi *et al.,* 2005; Adaramoye, 2010). These biological properties are linked to the high flavonoid (biflavanoids)

content of KV and therefore it has gained considerable interest in the treatment of disease (Adaramoye *et al.,* 2005).

Several investigations using KV have shown potential in the prevention of OS in the male reproductive system (Adaramoye *et al.,* 2012a; 2012b). The ability of KV to inhibit oxidation is due to its properties to scavenge ROS including $O_2^-$, $1O_2$, $OH^-$ and $LOO^-$ in animal experimental models (Farombi *et al.,* 2002; Farombi *et al.,* 2004a). Kolaviron regulates interconnected endocrine glands as well as the immune and nervous systems; protects the neurons of the brain against OS (Oluyemi *et al.,* 2007; Ofusori *et al.,* 2008). Moreover, KV influences testicular function; steroidogenesis and spermatogenesis (Akpantah *et al.,* 2003; Oluyemi *et al.,* 2007; Ofusori *et al.,* 2008).

There has been an increased focus on the role of OS associated with male infertility (Aitken *et al*., 2004; Amaral *et al.,* 2008; Badade and Samant, 2011). The research on natural products with the potential to reduce OS in the testis and improve seminal qualities is currently on the rise. Kolaviron has been shown to ameliorate testicular toxicity caused by nevirapine (Adaramoye *et al.,* 2012a), ethanol induced toxicity (Adaramoye *et al*., 2013), gamma irradiation (Adaramoye *et al.,* 2012b), di-n-butyl-phthalate (Farombi *et al.,* 2007) and atrazine (Abarikwu *et al*., 2012) in Wistar rats. In these studies the protective effects observed was also associated with a reduction in ROS and MDA and an improvement in antioxidant activity. Adaramoye and Lawal (2013) reported that KV pocesses antidiabetic and antioxidant properties to treat both DM and male infertility.

Additionally, the antioxidant activity of KV makes it suitable to be used as chemopreventive in degenerative diseases like cancer via biochemical mechanisms due to the protection against OS damage, up-regulation of phase carcinogen detoxifying enzymes, metal chelating activity, inhibition of cancer cell proliferation, inhibition of stress response proteins, down regulation of transcription factors and stabilization of membrane activity (Farombi and Owoeye*,* 2011).

# CHAPTER THREE

# RESEARCH DESIGN AND METHODOLOGY

## 3.1. Animal treatment and ethical clearance

Adult male Wistar rats, weighing about 240–290 g were randomly placed into 5 groups consisting of 12 rats each. The first group served as a normal control, while the second group served as the STZ induced diabetic control group. The third group included normal rats supplemented with KV (100mg/kg), while the fourth group was STZ induced diabetic rats also supplemented with KV (100mg/kg) five times per week for a period of six weeks. The final group included STZ induced diabetic rats given subcutaneous injections of 0.2IU /kg of insulin every second day.

Animals were accommodated individually in plastic cages at the animal facility of the Medical Research Council, South Africa. They were supplied with water and standard rat feed ad libitum. Animals were maintained under standard laboratory conditions at 22 ± 2°C with a 12-h light/dark cycles and humidity at 55 ± 5%. Body weights were measured from onset of the study and monitored throughout the feeding period until sacrifice.

All animals received care according to the principles of Laboratory Animal Care of the National Society of Medical Research and the National Institutes of Health Guideline for the Care and Use of Laboratory Animals of the National Academy of Sciences (National Institutes of Health publication no. 80-23, revised 1978). Ethics approval was obtained from the Faculty of Health and Wellness Sciences Ethical Committee at the Cape Peninsula University of Technology (Cape Town, South Africa) (NHREC: REC-230408-014).

After collecting samples following the sacrifices, all assays were done in the Oxidative Stress Laboratory of the Cape Peninsula University of Technology, Health and Wellness Sciences, Biomedical Sciences.

### 3.2 Study design

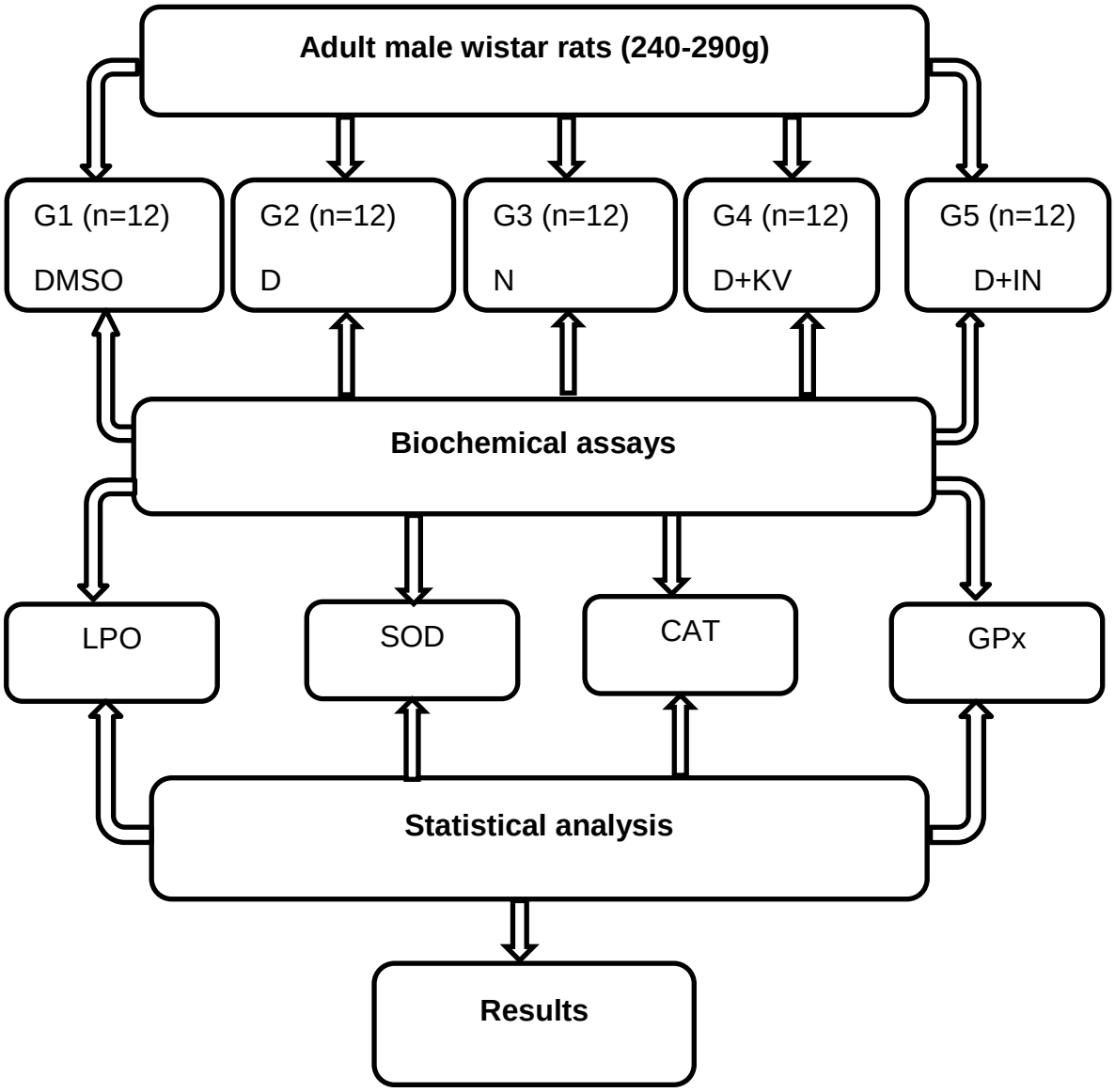


**Figure 3. 1: Schematic representation of the experimental design**
***Abbreviations*:** G1: First group, G2: Second group. G3: Third group, G4: Fourth group, G5: fifth, N= N=non-diabetic group, D: diabetic group, KV: kolaviron, IN: insulin, LPO: lipid peroxidation, SOD: superoxide dismutase, CAT: catalase, GPx: Glutathione peroxidase

The overall time period for the current study was 6 weeks. Rats were randomly divided in five groups (n=12 per group), each group received a specific diet treatment as specified below.

**Group 1 (non-diabetic control rats; N):** Dimethylsulfoxide (DMSO) only (vehicle for dissolving KV) was administered to normoglycemic rats by gastric intubation five times a week for 6weeks.

**Group 2 (diabetic rats; D)**: diabetic rats were given standard rat chow (SRC), DMSO and water for a period of 6 weeks.

**Group 3 (KV only (100mg/kg) treated; N+ KV)**: Normoglycemic rats were treated with 100mg/kg KV in DMSO (Adaramoye *et al*., 2005) by gastric intubation, five times a week for 6weeks.

**Group 4** (**D+KV**): diabetic rats were treated 100 mg/kg KV in DMSO, five times a week for 6 weeks.

**Group 5 (diabetic plus insulin (0.2**IU**/kg); D+IN)**: Diabetic rats were fed with standard rat chow (SRC), DMSO, water and were given subcutaneous (sc) injection of the standard anti-diabetic drug, insulin (0.2IU/kg) every second day for 6 weeks.

### 3.2.1 Plant materials and KV extraction

Fresh seeds of G kola were purchased from the Bodija market in Ibadan, Oyo State, Nigeria and authenticated by Professor E. Ayodele at the Department of Botany, University of Ibadan. A voucher specimen is available at University of Ibadan, at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan.

Kolaviron was extracted and isolated according to the method of Iwu *et al.* (1990). The seeds were peeled sliced and air dried (25–28°C). Briefly, the powdered seeds were extracted with light petroleum ether (bp 40–60°C) in a soxhlet for 24 hours. The defatted dried product was packed and extracted with acetone. The extract was concentrated and diluted twice its volume with water and extracted with ethylacetate (6×300 ml). The concentrated ethylacetate yielded a golden yellow solid termed KV.

### 3.2.2 Induction of diabetes

The animals were fasted overnight and diabetes was induced by a single intraperitoneal injection of freshly prepared STZ solution (Sigma, USA) at 50mg/kg body weight, dissolved in 0.1 M cold citrate buffer, pH4.5 (Atilla *et al*., 2012). Five days after STZ injection, blood glucose levels were measured by a portable

glucometer (Accu-Chek, Roche, Germany) in blood collected from the tail and diabetes status was confirmed when glucose level was above 18mmol/l.

### 3.3 Sample collection and preparation

At completion of the treatment periods rats were weighed and anaesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Fasting blood glucose level was measured after 4 hours of fasting (usually between 10 am and 2 pm). Blood samples were collected from the abdominal aorta into glucose tubes (containing sodium fluoride/potassium oxalate), EDTA-containing tubes and serum clot activator tubes. The epididymis and testes were also excised and weighed. The tissue samples were snap frozen in liquid nitrogen and stored at -80$^{\circ}$C.

Prior to analysis, tissue samples were thawed and 50mg of the testis and epididymis was placed in a glass homogenizer on ice. Briefly, 250µl of phosphate buffer (50mM $NaH_2PO_4.2H_2O$, 0.5% (v/v) TritonX-100, pH7.5) was added to 50mg of tissue. The homogenates were transferred into tubes and centrifuged at 10000rpm for 10min at 4$^{\circ}$C. The supernatants were subsequently transferred to new tubes and kept at -80$^{\circ}$C until used.

#### 3.3.1 Determination of protein concentration

Protein contents of the tissue samples were determined using the Bicinchoninic Acid (BCA) method. The principle of the BCA protein assay relies on the formation of a $Cu^{2+}$ protein complex under alkaline conditions, followed by a reduction of the $Cu^{2+}$ to $Cu^{1+}$ (Smith,1985). The amount of reduction is proportional to the amount of protein present. Bicinchoninic Acid forms a purple-blue complex with $Cu^{1+}$ in alkaline environments, thus providing a basis to monitor the reduction of alkaline $Cu^{2+}$ by proteins. This water-soluble complex exhibits a strong absorbance at 562nm.

Accordingly, protein concentrations generally are determined and reported with reference to standards of a common protein such as bovine serum albumin (BSA). A series of dilutions of known concentration were prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown was

determined based on the standard curve. Briefly, BCA working reagents, samples and standards were prepared referring to the manufacturer's instructions for the assay Kit supplied by Sigma Aldrich. Diluted samples (10 times) were run in triplicate assay; 10 µl of each standard or unknown sample were dispersed into a microplate well, 200 µl of the working reagent were added to each well and the plates were covered and incubated at 37°C for 30 minutes. The absorbance was measured at 562nm on a microplate reader (Thermo Electron Corporation, Multiskan spectrum, USA) and the protein concentration were quantified by using the standard curve and expressed as µg/ml.

### 3.4 Biochemical experiments

#### 3.4.1 Lipid peroxidation

Malondialdehyde levels were determined in the samples through a modern HPLC based thiobarbituric acid (TBA) assay method. This method is highly specific because it quantifies the genuine MDA-$(TBA)_2$ adduct formed (Lykkesfeldt, 2001). The quantitative analysis of MDA was performed using a modified method of Cuny *et al.* (2004) on a Spectra SYSTEM$^{TM}$ HPLC (Agilent technology, 1200 series, Germany). The principle of this method consists on the reaction of MDA with TBA in acidic conditions and at a higher temperature to form a pink MDA-$(TBA)_2$ complex in which the MDA content was quantified chromatographically. The concentration of MDA in samples as an index of lipid peroxidation was determined by HPLC with UV light at excitation of 515 nm and emission at 553nm.

Briefly, 50µl of sample was mixed with 375µl orthophosphoric acid 0.44M, 125µl thiobutric acid and 225µl distilled water. This mixture was heated at 100$^{\circ}$C for 60minutes and cooled down on ice. There after 775µl of alkaline methanol was added and the sample was subsequently vortexed and centrifuged at 3500rpm for 3min at 4$^{\circ}$C. The supernatant (1ml) was collected; 500µl of n-hexane was added and centrifuged at 14000rpm for 2 minutes. The supernatant (500µl) was collected in chromatographic tubes and injected into the HPLC system. The readings were performed after 10 minutes and sample concentration MDA levels were expressed in µmol/g of tissue.

### 3.4.2 Superoxide dismutase (SOD) activity

Superoxide dismutase activity was determined by a modified method from Ellerby and Bredesen (2000). This assay uses 6 hydroxydopamine (6-HD) and diethylenetriaminepentaacetic acid (DTAPAS) to generate superoxide anions. However, in the presence of SOD, these superoxide anion concentrations are reduced, yielding less colorimetric signal. Briefly, samples were run in duplicate, in a 96-well plate, 15µl of 6-HD was added to 6µl of supernatant. An amount of 170µl of DETAPAC solution (0.1mM in SOD assay buffer (0.4mg in 10ml) was added to the mixture in the previous step and readings were taking immediately at 490nm for 4min at 1min intervals. The activity of SOD was calculated from a linear calibration curve and expressed as µmol/mg protein.

### 3.4.3 Catalase (CAT) activity

The CAT activity was assessed by measuring the amount of substrate, $H_2O_2$, remaining after sample addition (Aebi, 1984). The CAT induced decomposition of hydrogen peroxide into water and oxygen. The rate of disintegration is proportional to the concentration of CAT activity. The CAT activity was determined by measuring the change in absorbance of hydrogen peroxide and sample mixture. Briefly, the CAT assay was performed in duplicate; 150 µl $H_2O_2$ was added to 20µl of sample. Readings were determined by using a spectrophotometer (Thermo Electron Corporation, Multiskan spectrum, USA) at 240nm wavelength. The CAT activity was expressed as µmol/mg of protein.

### 3.4.4 Glutathione peroxidase (GPX) activity

The activity of GPx is derived from the oxidation of reduced β-ND(P)H in a conjugated glutathione reductase (GR) system using hydrogen peroxide (12mM) as a substrate. Glutathione peroxidase reacts with hydrogen peroxide oxidizing reduced glutathione (GSH) to Oxidized glutathione (GSSG). With GR and NADPH reduce GSSG to GSH in the samples and the consumption of NDPH can be to the peroxide content in sample testing. In brief, the GPx assay was performed in duplicate in a 96-

well UV Costar plate. Each well contained; 215µl assay buffer (AB: 50mM potassium phosphate, 1mM EDTA, pH 7.0), 5µl GSH (30.7mg/ml in water), 5µl GR (0.1U/ml in AB), 20µl of sample, and 5µl NAD (P) H was added to the mixture. Two readings were recorded (modified method of Ellerby and Bredensen, 2000). The first was the background of oxidation at 340nm for 3min in 30sec intervals for samples ($A_1$) and blank ($A_{1b}$). The second reading was performed after adding 50µl $H_2O_2$. This reading monitored the decrease of $H_2O_2$ due to NAD(P)H oxidation at 340nm for 2min . The GPX activity was expressed in µmol /mg of protein.

### 3.5 Statistical analysis

Data are expressed as mean ± standard deviation (Mean+SD). One-way analysis of variance (ANOVA) was used to test for significance between the groups. The Bonferroni Multiple Comparison analysis was used to compare the differences between the groups. Statistical analysis of control and D group before initiation of treatment was performed by unpaired student's t-test. Differences were considered significant at $P<0.05$. The GraphPad PRISM 5 software package and Microsoft Excel 2010 were used for all statistical evaluations and graphical representations.

# CHAPTER 4

# RESULTS

## 4.1. Plasma glucose levels in diabetic and non-diabetic groups before initiation of treatment.

Figure 4.1 shows the fasting plasma glucose levels in both non-diabetic (N) and diabetic (D) groups before the start of KV and insulin (IN) treatments. The average fasting glucose level was significantly higher in the D group compared to the N group (28.19 ± 2.25mmol/L versus 9.93 ± 0.51mmol/L, $p<0.05$).

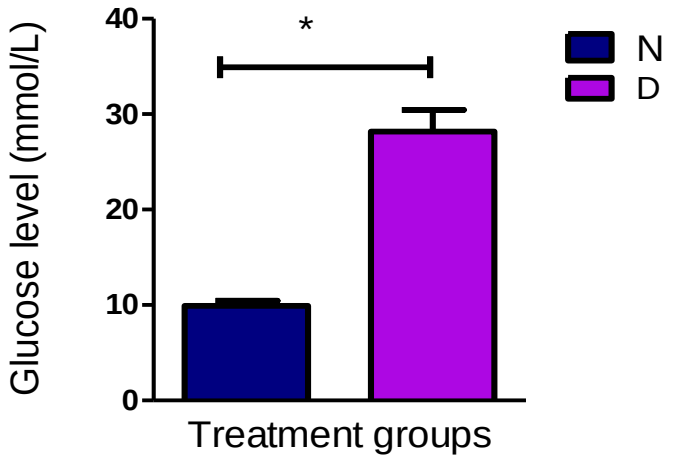


**Figure 4. 1: Glucose levels in diabetic and non-diabetic rats before KV and IN treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with $p<0.05$, N: non-diabetic control group, D: diabetic group.

## 4.2. Evaluation of body weights after subjecting the rats to various treatments

From Figure 4.2, it can be seen that the average body weight of rats injected with STZ was significantly lower compared to the N group at the time of sacrifice (257.1g ± 24.12g versus 398.3g ± 32.44g, $p<0.05$). Treatment of non-diabetic rats with KV (N+KV) did not show a significant difference in body weight when compared to the N group (399.0g ± 33.56g versus 257.1g ± 24.12g, $p>0.05$). There was a significantly higher body weight in rats from the N+KV group compared to the D group (399.0g ±

33.56g versus 257.1g ± 24.12g, p<0.05). In addition, a significant higher body weight observed in the D+KV group when compared to the D group (311.3g ± 33.06g versus 257.1g ± 24.12g, p<0.05). Furthermore, there was a significantly lower body weight in the animal injected with STZ and supplemented with KV (D+KV) group compared to the N group (311.3g ± 33.06g versus 398.3g ± 32.44g, p<0.05). Animals injected with STZ and supplemented with IN (D+IN) had a significantly lower body weight compared to N group (309.9g ± 25.96g versus 398.3g ± 32.44g, p<0.05). Furthermore, rats injected with STZ and insulin (D+IN) showed significantly higher body weight when compared to the D group (309.9g ± 25.96g versus 257.1g ± 24.12g, p<0.05). There were no significant difference in the total body weight between D+KV and D+IN groups (311.3g ± 33.06g versus 309.9g ± 25.96g , p>0.05)

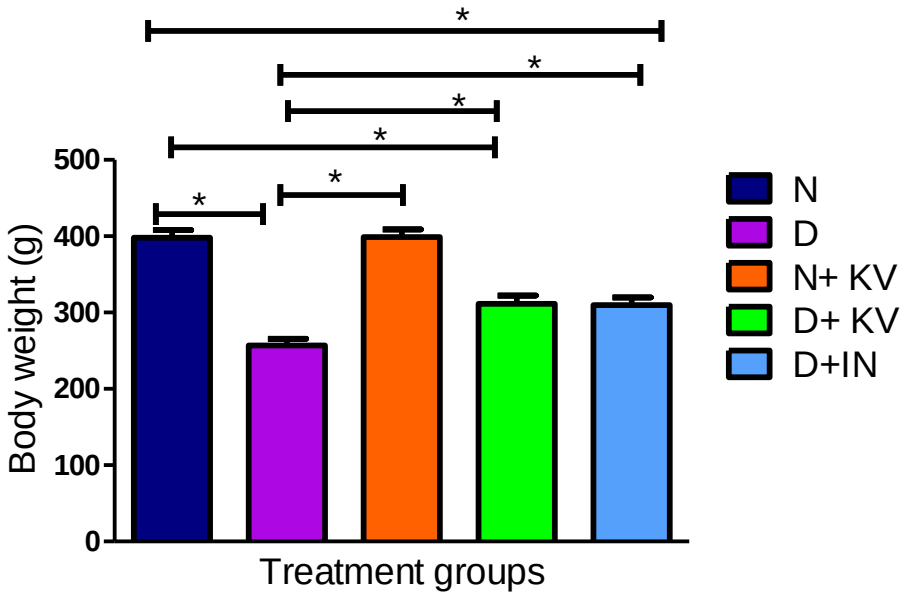


**Figure 4. 2 : Body weights of rats subjected to various treatments**

Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05. N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

### 4.3. Evaluation of testicular and epididymal weights of rats subjected to various treatments

Figure 4.3 represents the testicular weights of rats treated with STZ, KV and/or IN. The D group showed a significantly lower testicular weight when compared to the control N group (2.938g ± 0.421 g versus 3.63g ± 0.15g, $p<0.05$). No significant difference of testicular weight was observed in the N+KV group when compared to the N group (3.67g ± 0.35g versus 3.63g ± 0.15g, $p>0.05$). However, the testicular weights of rats fed with KV (N+KV) were significantly higher compared to D group (3.67g ± 0.35g versus 3.447g ± 0.197g, $p<0.05$) .The testicular weights of rats subjected to a double treatment; STZ and KV (D+KV) were significantly higher compared to D group (3.447g ± 0.197g versus 2.938g ± 0.421g, $P<0.05$). Testicular weights of rats from D+KV group was not significantly different (3.468g ± 0.197g versus 3.63g ± 0.150g, $p>0.05$) when compared to the N group. Rats treated with STZ and IN (D+IN) showed a lower significant testicular weight when compared to the N group (3.218g ± 0.1375g versus 3.630g ± 0.150g), however, there was no a significant difference when the testicular weights of the of the STZ+IN group was compared to the D group (3.218g ± 0.1375g versus 2.938g ± 0.421g, $p>0.05$). Furthermore, there was no significant difference in the testicular weights of rats when compared between the D+KV and D+IN groups (2.941g ± 0.89g versus 3.218g ± 0.1375g, $p>0.05$).

Figure 4.4 represents epididymal weights of rats subjected to treatments with STZ, KV and/or IN. Rats injected with STZ had a lower epididymal weight compared to the N group (0.431g ± 0.062g versus 0.529g ± 0.058g, $p<0.05$). The rats from the N+KV group did not show significantly differences in epididymal weight when compared to the N group (0.475g ± 0.09g versus 0.529g ± 0.058g, $p>0.05$), but a significantly higher epididymal weight was evident in the N+KV group when compared to D group (0.475g ± 0.09g versus 0.431g ± 0.062g, $p<0.05$). The epididymal weights of the D+KV group was not significantly different when compared to the N group (0.475g ± 0.088g versus 0.529g ± 0.0576g, $p>0.05$). A significantly lower epididymal weight in diabetic animal treated with IN compared to the N group was observed (0.454g ± 0.050g versus 0.529g ± 0.058g, $P>0.05$). However, no significant difference was found when compared to the D group (0.454g ± 0.050g versus 0.431g ± 0.062g,

p>0.05). Furthermore, there was no significant difference between D+KV and D+ IN groups (0.475g ± 0.088g versus 0.465g ± 0.048g, p>0.05).

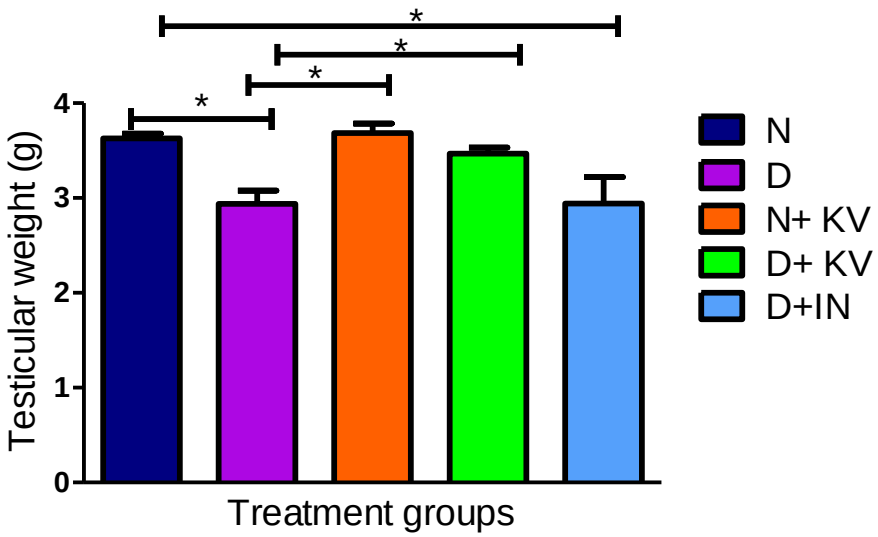


**Figure 4. 3: Testicular weights of male Wistar subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

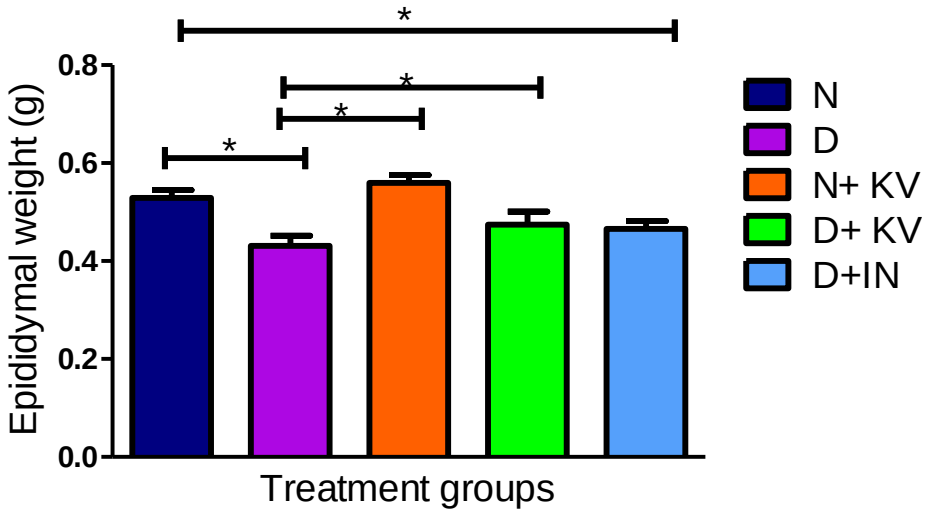


**Figure 4.4: Epididymal weights of male Wistar rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

**4.4. Assessment of lipid peroxidation of testicular and epididymal tissues of rats subjected to various treatments.**

Malondialdehyde levels in the testis are presented in Figure 4.5 for both non-diabetic and diabetic groups treated with KV or insulin. The testicular MDA level was significantly higher in the D group compared to the N group (0.014µmol/g ± 0.001µmol/g versus 0.010µmol/g ± 0.002µmol/g, $p<0.05$). The MDA level in the testes of non-diabetic rats fed with KV (N+KV) was significantly lower when compared to the N group (0.008µmol/g ± 0.001µmol/g versus 0.010µmol/g ± 0.002µmol/g, $p<0.05$). Furthermore, there were significantly lower testicular MDA levels in the N+KV group of rats compared to the D group (0.008µmol/g ± 0.001µmol/g versus 0.014µmol/g ± 0.001µmol/g, $p<0.05$). The diabetic testicular rats treated with KV (D+KV) showed significantly lower testicular MDA level than the N group (0.007µmol/g ± 0.001µmol/g versus 0.010µmol/g ± 0.002µmol/g, $p<0.05$). The testicular MDA levels in the D+IN group did not show a significant change when compared to the N group (0.012µmol/g ± 0.001µmol/g versus 0.010µmol/g ± 0.002µmol/g, $p>0.05$). However, animals treated with D+IN showed significantly lower LPO levels when compared to the D group (0.011µmol/g ± 0.001µmol/g versus 0.014µmol/g ± 0.001µmol/g, $p<0.05$). Moreover, the testes of animals injected with STZ and fed with KV (D+KV) produced significantly lower LPO levels compared to D+IN group (0.007µmol/g ± 0.001µmol/g versus 0.012µmol/g ± 0.001µmol/g, $p<0.05$).

Figure 4.6 shows the MDA level in epididymis of both non-diabetic and diabetic groups treated with KV and/or insulin. The MDA level was significantly higher in the D group compared to the N group in epididymal tissue (0.009µmol/g ± 0.004µmol/g versus 0.006µmol/g ± 0.002µmol/g, $p<0.05$). No significant differences in LPO levels was observed in the epididymal tissue of rats supplemented with KV (N+KV) compared to the non-diabetic (N) rats (0.006µmol/g ± 0.004µmol/g versus 0.006µmol/g ± 0.002µmol/g, $p>0.05$) and to the D group (0.006µmol/g ± 0.004µmol/g versus 0.009µmol/g ± 0.004µmol/g, $p>0.05$). Furthermore, a significant low epididymal MDA level was observed in the D+KV group when compared to the D group (0.006µmol/g ± 0.001µmol/g versus 0.009µmol/g ± 0.004µmol/g, $p<0.05$). In addition, no significant difference in epididymal MDA level was observed between

D+KV group and N group (0.006µmol/g ± 0.001µmol/g versus 0.005µmol/g ± 0.002µmol/g, p>0.05). Also, no significant difference in epididymal MDA level was observed in between D+IN group and N group (0.006µmol/g ± 0.002µmol/g versus 0.006µmol/g ± 0.002µmol/g, p>0.05). Animals treated with D+IN showed a significant low expression of MDA level when compared to the D group in the epididymal tissue (0.006µmol/g ± 0.002µmol/g versus 0.009µmol/g ± 0.004µmol/g, p<0.05). Moreover, no significant difference in epididymal MDA level was observed between D+KV and D+IN groups (0.006µmol/g ± 0.001µmol/g versus 0.006µmol/g ± 0.002µmol/g, p>0.05).

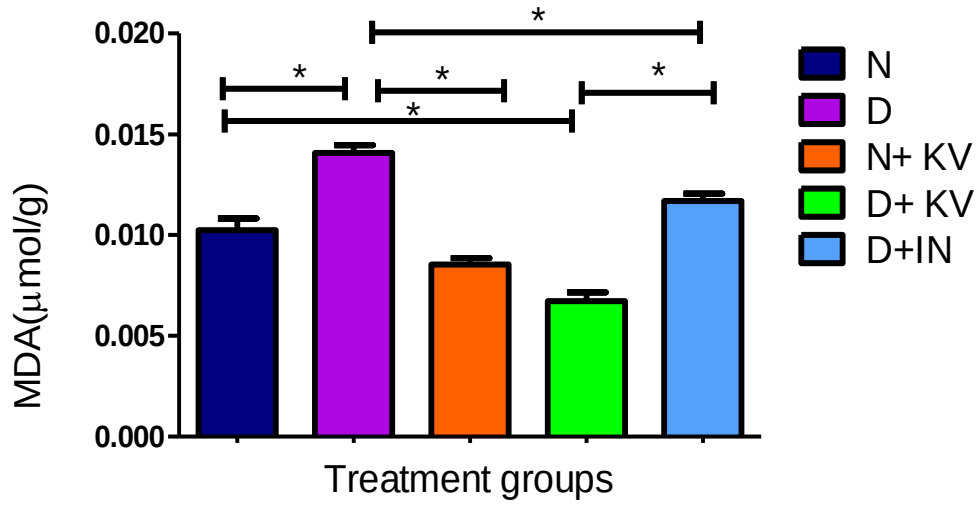


**Figure 4. 5: Testicular tissue lipid peroxidation levels of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05,. N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

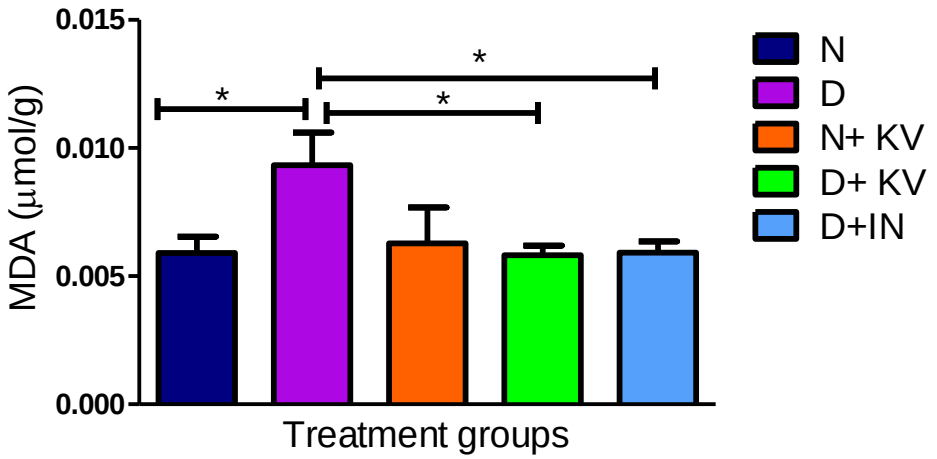


**Figure 4. 6: Epididymal tissue lipid peroxidation levels of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with $p<0.05$, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

### 4.5 Assessment of antioxidant enzymes in the testicular and epididymal tissue

Antioxidant enzymes have been investigated during the management of STZ induced diabetic rats in testicular and epididymal tissues. Various treatments such as KV or IN were used as supplementation and therapeutic.

#### 4.5.1 Assessment of SOD activity in testicular and epididymal tissue of rats subjected to various treatments.

The testicular SOD activity of rats treated with STZ, KV or IN is shown in Figure 4.7. No significant difference was observed in testicular SOD activity across all treatment groups.

Figure 4.8 represents the SOD activity in the epididymis of rats treated with STZ, KV and/or IN. In the epididymal tissue, the SOD activity was significantly low in the diabetic (D) group when compared to the N group (0.042µmol/mg ± 0.007µmol/mg, $p<0.05$ versus 0.556µmol/mg ± 0.007µmol/mg, $p<0.05$). On the other hand, there was no significant difference in the epididymal SOD activity of the D+KV group when compared to the N group (0.051µmol/mg ± 0.0011µmol/mg versus 0.056µmol/mg ± 0.007µmol/mg, $p>0.05$). Similarly, no significant difference was observed in the D+IN

group when compared to the N group (0.053µmol/mg ± 0.006µmol/mg versus 0.056µmol/mg ± 0.007µmol/mg, p>0.05). Moreover, the non-diabetic group supplemented with KV (N+KV) did not show any significant difference in the epididymal SOD activity when compared to the N group (0.053µmol/mg ± 0.009µmol/mg versus 0.056µmol/mg ± 0.007µmol/mg, p>0.05). However, the SOD activity of N+KV group was significantly higher than the SOD activity observed in the D group (0.053µmol/mg ± 0.009µmol/mg versus 0.042µmol/mg ± 0.007µmol/mg, p<0.05). Likewise, a significantly high epididymal SOD activity was observed in the diabetic rats injected with KV (D+KV) when compared to the D group (0.051µmol/mg ± 0.0011µmol/mg versus 0.042µmol/mg ± 0.007µmol/mg, p<0.05). In addition, a significantly high epididymal SOD activity was observed in diabetic rats treated with IN (D+IN) when compared to the D group (0.053µmol/mg ± 0.006µmol/mg versus 0.042µmol/mg ± 0.007µmol/mg, p<0.05, p<0.05). Furthermore, there was no significant difference between the D+KV group and the D+IN group (0.051µmol/mg ± 0.0011µmol/mg versus 0.053µmol/mg ± 0.006µmol/mg, p>0.05).

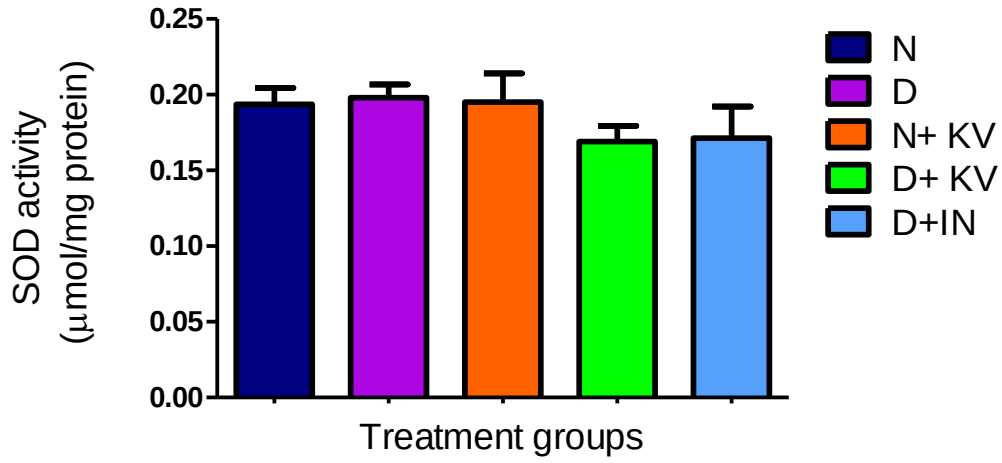


**Figure 4. 7: Testicular SOD activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05. N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

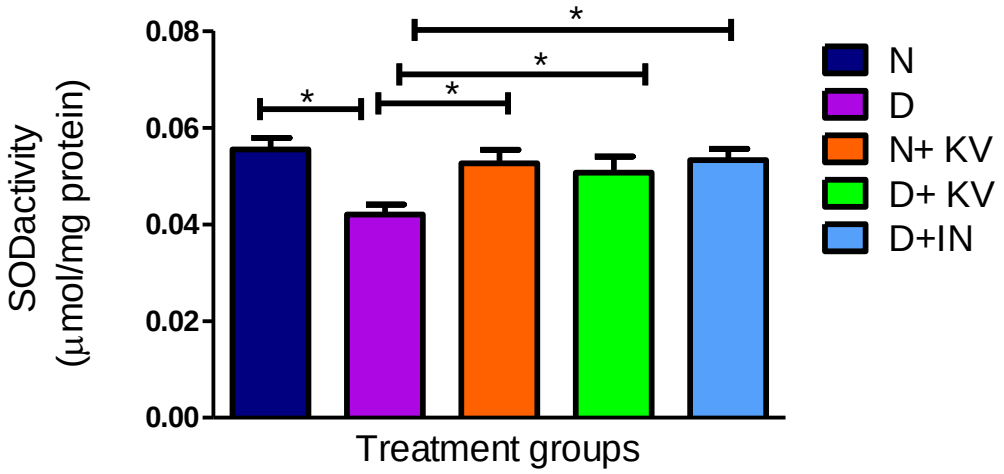


**Figure 4. 8: Epididymal tissue SOD activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

### 4.5.2 Assessment of the catalase activity in testicular and epididymal tissues of male Wistar rats subjected to various treatments

The CAT activity in the testes of rats treated with STZ, KV and or IN was shown in Figure 4.9. The STZ induced diabetic rats showed significantly low CAT activity in testicular tissue compared to N group (12.21µmol/mg ± 1.235µmol/mg versus 18.00µmol/mg ± 1.524µmol/mg, p<0.05). The supplementation of KV to non- diabetic rats (N+KV) did not show significant difference in the testicular CAT activity compared to the non- diabetic N control group (16.44µmol/mg ± 3.476µmol/mg versus 18.00µmol/mg ± 1.524µmol/mg, p>0.05). Likewise, the CAT activity of non-diabetic rats fed with KV (N+KV) was not significantly different from the diabetic (D) group (16.44µmol/mg ± 3.476µmol/mg versus 12.21µmol/mg ± 1.235µmol/mg, p>0.05). A significant testicular CAT activity was observed in diabetic rats treated with KV (D+KV) when compared to diabetic rats (D) (17.29µmol/mg ± 1.255µmol/mg versus 12.21µmol/mg ± 1.235µmol/mg, p<0.05). No significant difference was observed comparing this group (D+KV) to the N group (17.29µmol/mg ± 1.255µmol/mg versus 18.00µmol/mg ± 1.524µmol/mg, p>0.05). A significantly high testicular CAT activity was observed after insulin treatment in diabetic rats (D+IN) when compared to the D group (15.52µmol/mg ± 0.288µmol/mg versus 12.21µmol/mg ± 1.235µmol/mg, p>0.05). However, a significantly low testicular CAT

activity was observed in the D+IN group when compared to non-diabetic N control group (15.52µmol/mg ± 0.288µmol/mg versus 18.00µmol/mg ± 1.524µmol/mg, $p>0.05$). In addition, there was no significant difference in the testicular CAT activity of diabetic rats treated with KV (D+KV) or IN (D+IN) (17.29µmol/mg ± 1.255µmol/mg versus 13.77µmol/mg ± 0.641µmol/mg, $p>0.05$).

From Figure 4.10 it can be observed that the STZ induced diabetic rats showed significantly low CAT activity in epididymal tissue compared to the N group (2.864µmol/mg ± 0.415µmol/mg versus 6.162µmol/mg ± 0. 612µmol/mg, $p<0.05$). There was no significant difference in the epididymal CAT activity of rats supplemented with KV (N+KV) when compared to the N group (3.915µmol/mg ± 0.878µmol/mg versus 6.162µmol/mg ± 0.612µmol/mg, $p>0.05$). A significant high epididymal CAT activity was observed in N+KV group when compared to the D group (3.915µmol/mg ± 0.878µmol/mg versus 2.864µmol/mg ± 0.415µmol/mg, $p<0.05$). The treatment of diabetic rats with KV (D+KV) showed significantly higher epididymal CAT activity than the D group (4.896µmol/mg ± 1.019µmol/mg versus 2.864µmol/mg ± 0.415µmol/mg, $p<0.05$). Diabetic rats treated with KV (STZ+KV) showed significantly low epididymal CAT activity when compared to the non-diabetic N group (4.896µmol/mg ± 1.019µmol/mg versus 6.162µmol/mg ± 0.612µmol/mg, $p<0.05$). A significantly high epididymal CAT activity was observed in the diabetic rats treated with IN (D+IN) when compared to the D group (5.694µmol/mg ± 0.421µmol/mg versus 2.864µmol/mg ± 0.415µmol/mg, $p<0.05$). However, a significantly low CAT activity was observed in D+IN group when compared to the N group (5.694 ± 0.421µmol/mg versus 6.162µmol/mg ± 0.612µmol/mg, $p<0.05$). There was no significant difference in the epididymal CAT activity between diabetic rat treated with IN (D+IN) or KV (D+KV) (5.694µmol/mg ± 0.421µmol/mg versus 4.896µmol/mg ± 1.019µmol/mg, $p>0.05$).

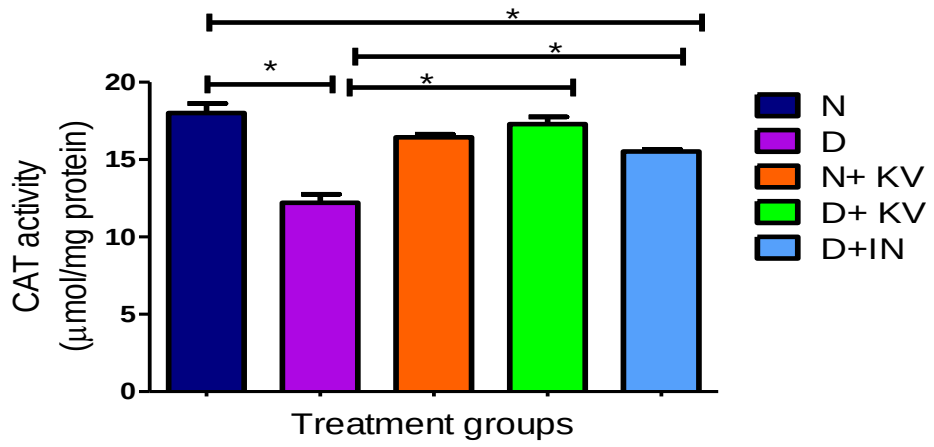


**Figure 4. 9: Testicular tissue CAT activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug)

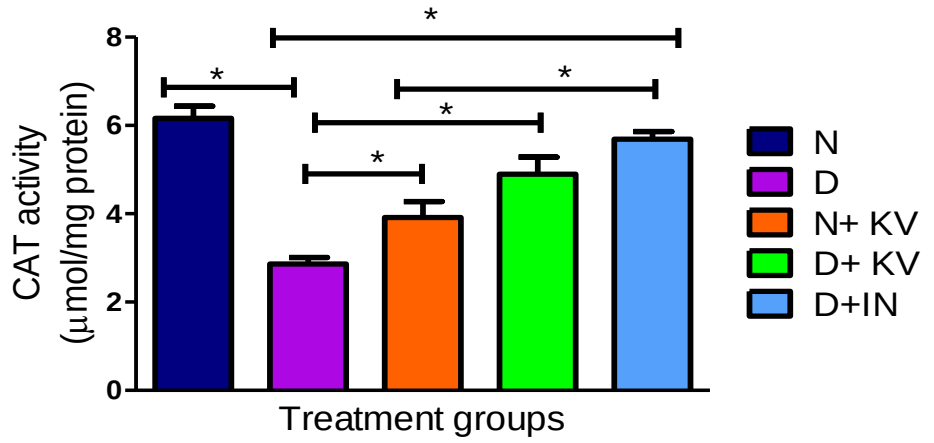


**Figure 4. 10: Epididymal tissue CAT activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05. N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug).

### 4.5.3. Assessment of the GPX activity in the testicular and epididymal tissues of male wistar rats subjected to various treatments.

The results of testicular GPX activity of rats treated with STZ, KV and/or IN is illustrated by Figure 4.11. A significantly low testicular GPx activity was observed in diabetic rats compared to the N group (3.977µmol/mg ± 0.880µmol/mg versus 12.26µmol/mg ± 0.644µmol/mg, p<0.05). The non-diabetic rats injected with KV

(N+KV) did not show any significant difference of the GPx activity in the testicular tissue compared to the N group (12.06µmol/mg ± 0.684µmol/mg versus 12.26µmol/mg ± 0.644µmol/mg, $p>0.05$). However, the testicular GPx activity of N+KV group was significantly higher than the GPx activity of the diabetic D group (12.06µmol/mg ± 0.684µmol/mg versus 3.977µmol/mg ± 0.880µmol/mg, $p<0.05$). The KV treated diabetic rats (D+KV) showed significantly high testicular GPx activity when compared to the D group (6.77µmol/mg ± 0.684µmol/mg versus 3.977µmol/mg ± 0.880µmol/mg, $p<0.05$). A significantly low testicular GPx activity was observed in diabetic rats treated with KV (D+KV) when compared to the non-diabetic N group (6.77µmol/mg ± 0.6844µmol/mg versus 12.26µmol/mg ± 0.644µmol/mg, $p<0.05$). In addition a significantly high testicular GPx activity was observed in the D+IN group when compared to the D group (8.006µmol/mg ± 0.7724µmol/mg versus3.977µmol/mg ± 0.880µmol/mg, $p<0.05$). Diabetic rats treated with IN (D+IN) showed significantly low testicular GPx activity compared to the N group (8.006µmol/mg ± 0.772µmol/mg versus 12.26µmol/mg ± 0.644µmol/mg, $p<0.05$). There was no significant difference testicular GPX activity between the diabetic rats treated with IN (D+IN) and KV (D+KV) (8.006µmol/mg ± 0.7724µmol/mg versus 6.77µmol/mg ± 0.6844µmol/mg, $p>0.05$).

Figure 4.12 represents the epididymal GPX activity of rats treated with STZ, KV and /or IN. Epididymal GPX activity was significantly low in diabetic rats compared to the N group (4.277µmol/mg ± 0.884µmol/mg versus 12.30µmol/mg ± 0.636µmol/mg, $p<0.05$). The supplementation of KV to non-diabetic rats (N+KV) did not show any significant difference in the GPx activity of epididymal tissue compared to the N group (11.81µmol/mg ± 0.700µmol/mg versus 12.30µmol/mg ± 0.636µmol/mg, $p>0.05$). However, N+KV group was significantly higher than the diabetic D group (11.81µmol/mg ± 0.700µmol/mg versus 4.277µmol/mg ±0.636µmol/mg, $p<0.05$). Epididymal GPX activity was significantly high in diabetic rats treated with KV (D+KV) compared to the D group (7.056µmol/mg ± 0.6571µmol/mg versus 4.277µmol/mg ± 0.636µmol/mg, $p<0.05$). The D+KV group showed significantly low epididymal GPx activity compared to the N group (7.056µmol/mg ± 0.6571µmol/mg versus 12.30µmol/mg ± 0.636µmol/mg, $p<0.05$). The treatment of diabetic rats with IN (D+IN) showed significantly high epididymal GPx activity compared to D group (8.006µmol/mg ± 0.810µmol/mg versus 4.277µmol/mg ± 0.636µmol/mg, $p<0.05$).

In addition, the (D+IN) group was significantly low when compared to the N group (8.006µmol/mg ± 0.810µmol/mg versus 12.30µmol/mg ± 0.636µmol/mg, p<0.05). No significant difference in the epididymal GPx activity was observed between the diabetic group treated with KV (D+KV) and the diabetic group treated with IN (D+IN) (7.056µmol/mg ± 0.657µmol/mg versus 8.006µmol/mg ± 0.810µmol/mg, p>0.05).

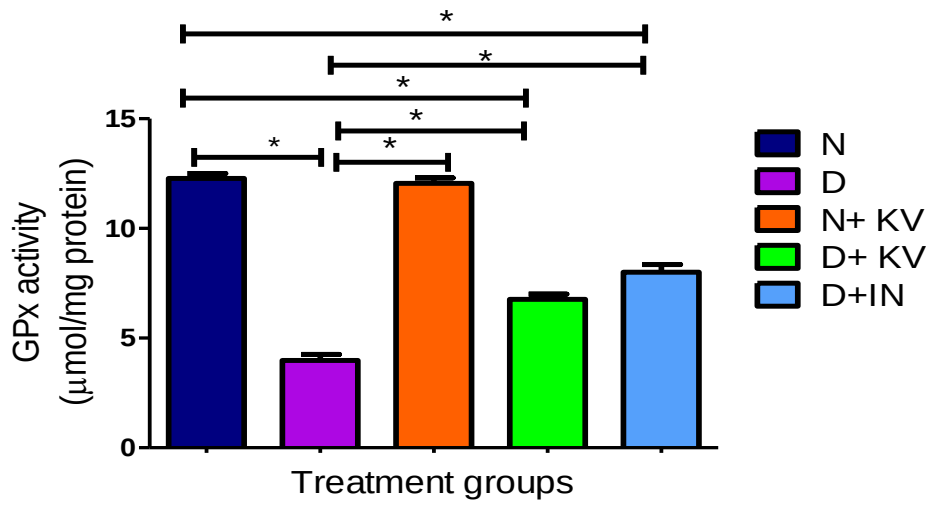


**Figure 4. 11: Testicular tissue GPx activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.**05**, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug)

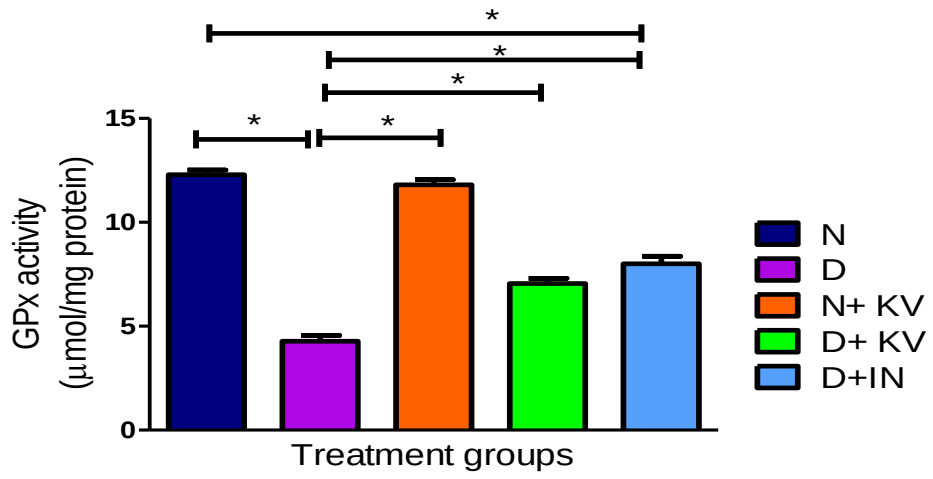


**Figure 4. 12: Epididymal tissue GPx activity of rats subjected to various treatments**
Data are presented as mean ± SD. (*) Indicates significant difference with p<0.05, N: non-diabetic control group, D: diabetic group, N+KV: non-diabetic group treated with kolaviron, D+KV: diabetic group treated with kolaviron, D+IN: diabetic group treated with insulin (standard drug)

# CHAPTER FIVE
# DISCUSSION

Diabetes associated with OS is said to impair testicular and epididymal tissue functions which can cause male infertility (Amaral *et al*., 2008). Increasingly, studies demonstrate the significant impact of phytochemicals such as flavonoids in the prevention and treatment of complications related to diabetes (Rahman, 2003; Rahimi *et al*., 2005). The physiological role and properties of flavonoids in the management of OS are currently being investigated in relation to male infertility. This interest is the motivation for the current study to investigate the effects of KV, a known-flavonoid extract of *G kola*, on testicular and epididymal induced OS using a diabetic rat model.

This study follows up on previous studies done in our laboratory using plant extracts such as red palm oil (RPO) (Aboua *et al*., 2009), rooibos (*Aspalathus linearis*) and green tea (*Camellia sinensis*) (Awoniyi *et al*., 2010). Rooibos and green tea extracts were shown to offer a protective effect against OS induced damage, thereby improving sperm quality and function (Awoniyi *et al*., 2010). Furthermore, in another study it was shown that dietary supplementation of RPO also reduced OS induced sperm damage (Aboua *et al*., 2009).

*Garcinia kola* was able to reduce the release of inflammatory and OS markers in both the blood (Ayepola *et al*., 2013) and ischaemia/reperfused heart tissue (Nyepetsi, 2014) of diabetic Wistar rats. Supplementing the diet with *G kola* improved the antioxidant and anti-inflammatory activities as well as cardiac functional recovery following ischaemia and reperfusion injury in isolated perfused rat hearts (Nyepetsi, 2014). In another study, Ayepola and colleagues (2013) demonstrated that the treatment of diabetic Wistar rats with an extract from *G kola* seeds (KV) ameliorated the blood plasma levels of proinflammatory cytokines and chemokines. With this in mind the aim of the current study was to determine the effects of KV on testicular and epididymal tissue in STZ induced diabetic Wistar rats. Various parameters such as blood glucose levels, body weights, LPO levels and activities of SOD, CAT, and GPx were determined. Additionally, the protective effets of KV have been observed in brain of Wistar albino rats exposed to gamma-radiation

induced OS (Adaramoye, 2010). Adaramoye and co-works, 2013 showed that KV might modurated testicular LPO by increasing antioxidant activities in ethanol-induced toxic wistar rats.

### 5.1. Evaluation of induced diabetes with STZ before kolaviron (KV) and insulin (IN) treatment.

Streptozotocin (STZ) is natural chemical compound used as chemotherapeutic agent in the treatment of cancer as well as used in medical research. Researchers around the world have used STZ to create experimental diabetes mellitus ( DM) because it is a simple, inexpensive and available method (Thulesen *et al*., 1997). Induction of DM by STZ is dose dependent and it should be two types; T1DM with large dose and T2DM with multiple low doses (Dufrane *et al.,* 2006). At high doses, typically given singly, STZ targets β cells by its alkylating property corresponding to that of cytotoxic nitrosourea compounds (Dufrane *et al.,* 2006). At low doses, generally given in multiple exposures, STZ elicits an immune and inflammatory reaction, presumably related with the release of glutamic acid decarboxylase autoantigens (Dufrane *et al.,* 2006).

Induction of diabetes with STZ decreases Nicotinamide-adenine dinucleotide (NAD) in pancreas islet beta cells and causes histopathological effects in beta cells which probably intermediates induction of diabetes (Akbarzadeh *et al.,* 2007). Furthermore, through the destruction of β cells associated with hyperglycemic conditions; thus initiate free radical production (Peschke *et al*., 2000; Hooda *et al*., 2014). Streptozotocin enters the β-cells and changes the DNA structure thereby activating ADP-ribosylation and leading to the depletion of cellular $NAD^+$ and ATP (Szkudelski, 2001). Dephosphorylation of ATP after STZ treatment produced superoxide radicals.

In the current study, the single intraperitoneal administration of STZ (50mg/kg) in adult wistar rats was effective in ablating the pancreatic β-cells and caused hyperglycaemia after 5 days. This was confirmed by the significantly higher plasma glucose levels (18mmol/l; see Figure 4.1) in the STZ group of animals which is typical of type1 diabetes mellitus. It was therefore concluded that the diabetic animal

model was successfully created and the results was similar to and supported by previous findings (Akbarzadeh *et al.,* 2007; Oyagbemi *et al*., 2014,) where induction of DM in wistar rats via intra-venous STZ injection (60mg/kg) was confirmed by hyperglycaemia after four days .

### 5.2. Evaluation of rat body, testicular and epididymal weights subjected to various treatments

Blood glucose levels is an indication of proper insulin function and important energy sources (Kraemer and Ratamess, 2005). Insufficient insulin secretion or dysfunction of the signalling pathway results in a disturbance of glucose homeostasis. Subsequently the body will start to use other macromolecules such as lipids and proteins as sources of energy (Ravi *et al*., 2004). This results in shrinking of muscle tissue accompanied by a rapid weight loss in diabetic animals (Abdel-Hamid, 2002; Ravi *et al*., 2004; Ayepola *et al.,* 2013). Data from the current study showed that the body, testes and epididymal weights were significantly lower in STZ induced diabetic rats compared to the non-diabetic control group (Figure 4.2, 4.3, 4.4). These findings are in agreement with previous studies that also demonstrated a significant decrease in body, testicular and epididymal weights in diabetic rats (Bal *et al*., 2011; Farombi *et al*. 2012). Moreover, variations in animal body and organ weights have been reported to affect spermatogenesis, sperm quality and sperm concentration (Griffeth *et al*., 2013).

Medicinal plants have been used to treat OS caused by different toxic substances and this influences the body and organ weights (Aitken and Roman, 2008; Farombi *et al*., 2012). In the present study, KV extract from *G kola* has been used as a potential antidiabetic and antioxidative supplement in comparison to the standard synthetic antidiabetic drug (insulin).

In the current study, diabetic animals treated with insulin improved their body and epididymal tissue weights as there was a clear regain of body weights (Figure 4.2, 4.4). Synthetic Insulin is a standard drug used to treat diabetes which is different from the insulin secreted naturally by the pancreas. Pancreatic insulin promotes proper metabolism, energy balance, and maintenance of normal body weights (Kanzaki and Pessin, 2001; Boura-Halfon *et al*., 2009). Though, the synthetic insulin

used in this study had improved the weight of diabetic animals, there was still not total recovery to their normal weights. This might be due to the destruction of some insulin receptors when the animals were injected with STZ. However, some receptors might allow the synthetic compound to enter the cells in order to alleviate the OS state in rats injected with STZ and administered with IN.

Likewise, comparison was statistically carried out on the effect of KV in the management of STZ induced diabetes and a similar response was observed. Supplementation with KV showed similarity to the IN treatment with a significant improvement not only of the diabetic rat's body and epididymal tissue weights, but also of the rat's testicular weight. Such findings are in agreement with the results of Adaramoye and Lawal (2013), who reported that the treatment with KV significantly increased the weight gained by diabetic rats when compared to the untreated diabetic counterparts. Moreover, the results of this study did not indicate a significant difference in weights of non-diabetic rats supplemented with KV (N + KV) when compared to non-diabetic control group (N) (Figure 4.2, 4.3, 4.4). These results demonstrate that KV supplementation had no adverse effects on the animal weights confirming that the decrease observed in diabetic rats supplemented with KV was only due to their diabetic condition (Figure 4.2, 4.3, 4.4). This also implies that the body, testes and epididymal weight improvement observed in the diabetic animals supplemented with KV might be due to its antioxidant and hypoglycemic potential to prevent OS and diabetes. Actually, previous studies have reported on the hypoglycemic potency of KV through different mechanisms such as insulin release from the pancreatic β cells and hepatic glucose storage via stimulation of glycolytic and glycogenic enzymes or inhibition of glucose-6-phosphatase (Mezei *et al*., 2003; Pinent *et al*., 2004; Ayepola *et al*., 2013). Therefore, it can be argued that the ability of KV to protect against weight loss might mainly be attributed to its glucose lowering capacity. Indeed, the regulation of glucose levels as the main source of energy by KV provides a platform for less use of alternative sources of energy from body, testicular, epididymal proteins and fats. Being a flavonoid-rich compound, whose action is directly related to the hypoglycemic potency, KV also counteracts OS through its antioxidant activity.

Altogether, these results demonstrate that KV supplementation can mimic IN symptomatic treatment of the animal weight. It could therefore be postulated that KV might worth been considered as an antidiabetic compound in the management of weight and glucose regulation in diabetes.

### 5.3 Assessment of lipid peroxidation of testicular and epididymal tissues of rats subjected to various treatments.

Malondialdehyde is an end product of LPO and the increased levels are an indication of oxidative damage (Avci *et al*., 2014). It has been shown that LPO induces disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, modifies low density lipoprotein (LDL) to proatherogenic and proinflammatory forms, and generates potentially toxic products (Greenberg *et al*., 2008). Thus LPO in vivo has been implicated as the underlying mechanisms in numerous disorders and diseases such as cardiovascular diseases, cancer, neurological disorders, and aging. The mechanism of free radical-mediated LPO reactions include (1) abstraction of bisallylic hydrogen from polyunsaturated fatty acids to give carbon-centered radicals which rearranges to more stable cis,trans-pentadienyl radicals, (2) addition of oxygen to the pentadienyl radical to give lipid peroxyl radicals, (3) release of oxygen from the peroxyl radical to give oxygen and pentadienyl radicals, which rapidly react with oxygen to give a thermochemically more stable trans,trans form preferentially than cis, trans form, and (4) intramolecular addition of the peroxyl radical to the double bond to yield bicyclic prostaglandin-type products (Greenberg *et al*., 2008).

The results clearly indicate asignificantly high and increased expression of MDA in the testicular (Figure 4.5) and epididymal (Figure 4.6) tissues of the diabetic rats when compared to the non-diabetic rats. These results are in agreement with those from a previous study also performed on diabetic experimental animal models (Adaramoye and Lawal, 2013). During diabetes, hyperglycemia causes auto-oxidation of glucose and stimulates OS through excessive free radicals production. The release of free radicals causes damage to biological systems by abstracting electrons from macromolecules thereby causing instability and disintegration. For instance, peroxidation of polyunsaturated lipids on sperm membrane has been

reported to cause structural alterations of the biological cell membranes as well as a change in membrane stability and function (Adaramoye and Arisekola, 2013). The peroxidation of sperm lipids may also disturb maturation, spermatogenesis, capacitation, acrosome reaction and eventually in membrane fusion which result in male infertility (Sanocka and Kurpisz, 2004).

Different flavonoid rich plant materials have been used to assess OS and its potential to protect male reproductive organ function (Luo *et al*., 2006; Ozyurt *et al*., 2006; Mallick *et al*., 2007; Aitken and Roman, 2008; Aboua 2009). For instance; the protective effects of caffeic acid phenethyl ester, a bioactive component derived from honeybee hive propolis, has been demonstrated in testicular tissues of cigarette smokers (Ozyurt *et al*., 2006). *Lycium barbarum* extract from Lycium fruit, has been shown to offer a protective effect against testicular OS caused by heat stress and hydrogen peroxide (Luo *et al*., 2006). An aqueous-methanol extract of *Musa paradisiaca, Tamarindus indica, Eugenia jambolana* and *Coccinia indica* (MTEC) has been shown to offer protection against the oxidative testicular damage resulting from induced diabetes (Mallick *et al*., 2007). The oligosaccharides extracted from wheat bran have also been shown to protect the testes from the OS associated with alloxan-induced diabetes in rats (Ou *et al*., 2007). Aboua *et al*. (2009) had reported that red palm oil was an excellent dietary supplement in reversing the testicular and epididymal antioxidants enzymes during tertiary-butyl hydroperoxide (t-BHP) induced OS. Oxidative stress epididymal Sperm treated with rooibos extracts improved its sperm quality and function with lower lipid peroxidation levels formation (Awoniyi *et al*., 2012).

In the present study, the protective mechanism of KV and IN has been examined in the onset of lipid peroxidation related to STZ induced diabetes. The findings from the study indicate that diabetic animals treated with IN recovered from LPO in the testes and epididymal tissues. This is observed through the decrease of MDA level to values close to baseline of non-diabetic rats (Figure 4.5, 4.6).

The effect of KV in the management of LPO was then compared to the effect of IN. The current study expected that KV as natural antioxidant could offer protective effect. Kolaviron showed signinificantly healthier responses. Not only did KV

supplementation significantly reduce MDA levels in the N+KV group when compared to N group, but it also had a better effect than IN in the restoration of testicular and epididymal MDA levels of the D+KV group when compared to D only group (Figure 4.5 ). Moreover, MDA results of all KV supplemented groups were significantly lower than the results of non-diabetic (control) rats. Therefore, the effect of KV on LPO is much appreciated to prevent diabetes. Collectively, these results demonstrate that KV supplementation helps to protect against OS. Furthermore, although both KV and IN restored LPO levels; these findings emphasize the potentiality of KV to better restore metabolic disorders related to OS such as diabetes and male infertility.

Insulin helps to control blood glucose levels, and then this reduces the amount of free radicals released. The observed protective effects of KV in this study may be due to its antioxidant properties by scavenging the effects of hydroperoxides resulting from induced-OS. This is in accordance with previous studies that have reported the beneficial effects of KV against testicular damage induced by various chemicals (Adaramoye *et al*., 2005; Adaramoye and Adeyemi, 2006; Adaramoye *et al*., 2013).

### 5.4. Assessment of antioxidant enzymes in the testicular and epididymal tissue to various treatments.

Antioxidant enzymes such as SOD, CAT and GPX play a crucial role in protecting the testes and epididymal tissues against OS associated damage and male reproductive disorders (Aitken *et al*., 2008, Ourique *et al*., 2013; Kokilavani *et al*., 2014). Physiological and pathophysiological conditions such as diabetes influence the level of production and activity of these antioxidant enzymes (Saxena *et al*., 1993; Maritim *et al* 1999). The reduction in antioxidant enzymes have been previously reported in diabetic (Erejuwa *et al*., 2011; Gupta *et al*., 2011; Ayepola *et al*., 2013). The observed reduction in antioxidant enzyme activities could be due to the oxidative inactivation of the enzyme by ROS or by the glycation of the enzymes (Pigeolet *et al*., 1990; Sozmen *et al*., 2001; Soon and Tan, 2002; Ravi *et al*., 2004). The reduced activity of SOD, CAT and GPX in the epididymal and testicular tissues have been observed following STZ induction of diabetes and this may result in a

number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides.

Superoxide dismutase catalyzes the dismutation of the $O_2^{\bullet-}$ to produce $H_2O_2$. This product is the substrate of CAT and GPx which both transform hydrogen peroxide into water and oxygen. Unlike the findings of Adaramoye and Lawal (2013) who reported lower SOD activity in the testes of diabetic rats; the current study, showed no significant differences in testicular SOD levels of STZ induced diabetic rats (Figure 4.7). The findings of our study could be based on the a few arguments. The dose and rate of administration of STZ did not induce a severe state of OS and diabetes. Although, the model of administration of STZ used in this current study was modified from those used by Atilla *et al*. (2012) and the dose of STZ injected in this current study was exactly the same as the dose used. In the current study, the fasting period before sacrifice could have also influenced the results. The rats were fasted for 4 hours. The fasting period varied greatly in several studies; for instance 3 hours (Atilla *et al*., 2011) and 24hours (Mallick *et al*., 2007).

In epididymal tissue the SOD activity was significantly lower in STZ-induced diabetic rats when compared to non-diabetic control groups (Figure 4.8). This is in agreement with a study by Adaramoye and Lawal (2013) which demonstrated that diabetogenic agent reduces SOD activity in epididymal tissue. Both catalase and GPx activities in the testes and epididymis were significantly lower in STZ induced diabetic rats than in the non-diabetic control rats (Figure 4.9, 4.10, 4.11, 4.12). Their activities are regulated by SOD activity. The current study shows the testicular CAT and epididymal CAT activity are 10 times SOD activities (Figure 4.9, 4.10). However, GPx activities are estimatated 5 times SOD activities (Figure 4.11, 4.12). Glutathione peroxidase shares the substrate, $H_2O_2$, with CAT; it alone can react effectively with lipids and other organic hydroperoxides, being the major source of protection against low levels of OS. Some authors supported the idea that GPx were essential in the protection against OS under normal conditions (Vernet *et al*., 2004). Others believed in a protective role for these enzymes only under OS conditions (Kelner and Bagnell, 1990). Generally, in our study the antioxidant enzymes before treatments have shown similar expression activity in testicular and epididymal tissues. In STZ-induced diabetes associated with OS, the antioxidant enzymes in testicular and

epididymal tissues were depleted. This is supported by the high expression of MDA levels and the concomitant reduction in endogenous antioxidant activities including SOD, CAT and GPx.

Numerous compounds with antioxidant activities have been shown to improve or normalize the activities of antioxidant enzymes in non-diabetic and diabetic rats respectively (Maritim *et al.,* 2003). In the present study, the supplementation of KV for six weeks to the normal rats did not change SOD, CAT and GPx activities compared to the non–diabetic rats. This might be due to the interference between natural antioxidants produced by the body and the natural antioxidant effects of KV. The treatment of STZ induced diabetic rats with KV influenced a high expression of SOD, CAT and GPx activities compared to the diabetic groups (Figure4.5, 4.6, 4.7, and 4.8). Similarly to previous studies, the supplementation of dietary antioxidants to experimental animals has shown a positive correlation between natural dietary supplementation and increased antioxidant enzyme levels in induced OS models. For example, significantly high activities of antioxidant enzymes was observed in oxidative stress induced diabetic rats supplemented with *Gymmema montanum* leaf extract (Ananthan and co-workers 2004). In other studies, KV restored antioxidant enzymes in the testes of diabetic induced rats (Farombi *et al*., 2012; Adramoye *et al.,* 2013). The protective effect of KV observed in the testes and epididymis of diabetic rats might either be due to the inhibition of glycation by the antioxidant enzymes or scavenging of ROS, thus decreasing the formation of LPO (Adaramoye *et al*., 2005; Farombi and Nwaokeafor, 2005).

Insulin has been used in the management of diabetes by restoring pancreatic insulin deficiency. In our investigation, it has been used to treat STZ induced diabetes in Wistar rats. From our results it is clear that synthetic insulin had improved the levels of testicular and epididymal antioxidant enzymes in STZ induced diabetic rats.
These results confirm the central role of insulin in energy homeostasis and also make it an important signalling factor in the reproductive tract (Obici *et al*., 2002). In the current study, statistical results have shown that synthetic IN had the same effect as KV. We therefore postulate that KV might has reduced, inhibited or alleviated the negative effects of STZ-induced diabetes on rat reproductive function because of its hypoglycaemic and antioxidant properties.

# CHAPTER SIX

## CONCLUSION AND RECOMMENDATIONS

In this study, STZ induced-diabetes alters testicular and epididymal tissue functions possibly by inducing OS resulting in increased LPO and disruption of antioxidant enzyme activities. We explored how KV could modulate OS biomarkers in an *in vitro* experimental animal model using Wistar rats. The findings of this study indicate that the supplementation of dietary KV could have a protective effect against STZ induced diabetes associated with OS.

The investigations performed in this study focused on using KV (biflavanoids) for supplementary and therapeutic treatments under normal and OS conditions in testicular and epididymal tissue of rats. The dietary KV treatment could have a protective effect against LPO damages by restoring MDA levels of OS and influencing antioxidant enzyme activities. Insulin produced a similar effect as KV. The improvement of antioxidant enzyme activities in diabetic rats was intended to overcome the effect of ROS. Increasing the intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system (Lobo *et al*., 2010).

We proposed that, dietary kolaviron treatment could have offered a protective effect against lipid peroxidation damages through scavenging of the released free radicals before they cause detrimental effects to the testicular and epididymal tissues. It was found that KV had the possibility of being used as a co-treatment for diabetes related pathologies and their complications.

The findings of this study emphasized the protective effects KV had on the male reproductive organs based on the levels of oxidative stress markers and antioxidant activities. Functional data such as motility and sperm count were not performed due to lack of proper infrastructure. This limited our data interpretation to antioxidants enzyme activities and MDA only. It could also be observed, that the small sample size and materials contributed to non-obtainment of clear results in all aspects of the study. Therefore, more studies with advanced technologies and larger sample sizes are recommended to further investigate the antioxidant properties of KV. We

propose further investigations to elucidate the effect of KV treatment directly to male reproductive organ functional activity and could be used to advance the current knowledge which could also be extended to clinical research.

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