CHAPTER ONE

1.0 Introduction

Infertility is a worldwide problem and most challenging issues among married couples (Agarwal & Prabakaran, 2005), even though it is not deadly, it can cause significant psychological trauma to an individual (Uadia & Emokpae, 2015). Infertility primarily refers to the biological inability to achieve pregnancy after one year of non-contraceptive, regular up to 3 to 4times per week, unprotected and active sexual intercourse (Cooper *et al*., 2010).

Survey carried out by Greil in the year 1997 revealed that approximately five percent of heterosexual couples have one or more unresolved infertility problem(s). However, 12 to 18% of couples experience unintentional barreness in a year (Chowdhury *et al*., 2017). Makar and Toth 2002 reported that infertility is cause by both male and female factors. Males are responsible for 20-30% infertility problems while females' poll 20-35% cases of infertility with 25-40% are as a result of combine problems from both. No cause is found in 10-20% of infertility cases (Makar and Toth 2002).

The chance to conceive depends on the length of sexual exposure, frequency of coitus, and couple's age. The normal, young aged couples have a 25% chance to conceive after 1 month of unprotected intercourse; 70% of the couple's conceive by 6 months, and 90% of the couples have a probability to conceive by 1 year. Only 5% of the couples will conceive after one and a half year or two years (Kakarla *et al*., 2008; Kamel, 2010).

Both males and females are equally responsible for the causes. Most of the infertile couples have one of these three major causes including a male factor, ovulatory dysfunction, or tubal-peritoneal disease (Kakarla *et al*., 2008). Moreover, medications have been reported to be one of the major causes of infertility, one of the groups of medications that causes infertility has reported by Xavier & William, 2010 is antipsychotic drugs. Antipsychotics also known as

neuroleptics are drugs used in the treatment of serious mental health conditions such as psychosis as well as other emotional and mental conditions (Araújo, 2014). Antipsychotic drugs are used mainly for the treatment of psychosis.

Psychosis is a serious psychiatric illness characterized by a deranged personality and a warped perception of reality, or an unstable state of the mind characterized by a lack of interaction with reality and marked by physical damage to the brain (Freudenreich, 2012). In layman's term psychotic individual could be described as someone who is "insane", "mad", "nuts" etc. (Gelder *et al*., 2005). Psychosis is a special type of mental disorder that affects around 2-3% of global population and has a strong genetic basis. The primary causes of psychosis include the following: Schizophrenia and schizophreniform disorder, schizoaffective disorder, mood disorder (which has manifestations of both schizophrenia and mood disorders), brief psychotic disorder, delusional disorder, and persistent hallucinatory insanity (Cardinal and Bullmore, 2011).

World Health Organization in 2001 estimated that, about 450 million people suffer from mental disorders and during ones lifetime, one out of every four people can experience one or more psychiatric or behavioral disorders. Meta-analytic studies report that among individuals living in cities, the prevalence of all psychiatric disorders is increased by 38%, mood disorders by 39%, and anxiety disorders by 21% as compared to inhabitants of rural areas (Peen *et al*., 2010). Schizophrenia and schizoaffective disorder account for over 60% of the prevalence of psychotic disorders. Majority of psychotic illnesses have their beginning in late adolescence or early adulthood (Peen *et al*., 2010).

According to a comprehensive analysis of all published incidence and prevalence studies of population rates of subclinical psychotic experiences, the median prevalence rate is about 5% and the median incidence rate is around 3%. A meta-analysis of risk factors associated with

mental disorder shows links to developmental level, child and adult social adversity, psychoactive substance use and migrant status, among other things. According to the slight variation between prevalence and occurrence rates, as well as evidence from follow-up research, roughly 75–90% of developmental psychotic experiences are transitory and fade away over time (Van-Os *et al*., 2009). However, depending on the degree of additional environmental risk the individual is exposed to, the transitory developmental expression of psychosis (psychosis proneness) may become abnormally persistent and clinically significant. The psychosis proneness–persistence–impairment model considers genetic background factors influencing a widely distributed and transitory population manifestation of psychosis during development, with poor prognosis expected by environmental exposure interacting with genetic risk in terms of persistence and clinical need (Van-Os *et al*., 2009). An estimated 20 % to 30 % of Nigeria population are believed to suffer from mental disorders (Onyemelukwe *et al*., 2016). Thus, mental illness is a seriuos ailment in our society which need urgent attention in term of management.

In the management of psychosis, antipsychotic medications are prescribed to individuals experiencing psychotic symptoms and are the primary treatment for patients with schizophrenia (Miyamoto *et al*., 2005). Antipsychotic drugs are also prescribed to those with depression and used in the treatment of traumatic brain injury and dementia and they work by blocking dopamine receptors (Muhammad and Abdolreza, 2008).

Antipsychotic drugs can be divided into a number of subtypes, based around when they were first synthesized or their constitutive receptor occupancy. Some of these antipsychotic drugs are Chlorpromazine, Reserpine, Haloperidol, Fluphenazine, Molindone and Loxapine which are first generation antipsychotic drugs and Clozapine, Asenapine, Olanzapine, Ziprasidone and Risperidone which are second generation antipsychotic drugs.

Chlorpromazine (CPZ) is a first-generation synthetic antipsychotic drug that is still widely used for the treatment of mental disorder around the world (Chong *et al*, 2004). Chlorpromazine is a dopamine antagonist from the antipsychotic drug class that also has antiadrenergic, anticholinergic, antihistaminergic and antiserotonergic effects (Healy, 2004). Chlorpromazine (CPZ) is on the World Health Organization list of essential medicines which remains drug of first choice for the treatment of schizophrenia despite the emergence of other drugs (WHO, 1998). It is a dimethylamine derivative of phenothiazine, has a chemical formula of $C_{17}H_{20}CN_2S$ and molecular weight: 355.321 g/mol. In the blood of warm-blooded, it interacts well with plasma proteins, its protein binding reaches 90-99%. The time of its half life was 30 ± 7 hours, which indicates its long-term stability.

Chlorpromazine was marketed under the trade names Thorazine and Largactil among others. Chlorpromazine (10H-phenothiazine-10-propanamine, 2-chloro-N, N-dimethyl) is a phenothiazine derivative. It is white crystalline solid and very soluble in ethanol, ether, benzene and chloroform (Anz *et al*., 2011). Its melting point is approximately 60^0 Cwith a boiling point of 200-205 degree (at 0.1KPa) and with a relative vapor pressure 0.7x10- 3 Pa (at 25^0 C estimated) (Penouka *et al*., 2011). Chlorpromazine is well absorbed through the mouth, with a maximum effect of around three hours and a long lasting effect. Intramuscular injection induces sedation in 30 minutes that lasts four to five hours, while intravenous injection produces results in five to ten minutes. However, repeated intramuscular administration may cause pain, leucocytosis, local induration and fever. Just around 8% of the total dose is excreted, which may happen quickly with convulsions or take longer if muscular hypotonia is serious. In a man, an attempt at suicide was followed by a full recovery in three days after taking 7025 mgm. tablets (i,750 mgm).

The use of herbal medicine in this present century cannot be overlook, there is a resurgence of herbal medicine as people want more control in their personal healthcare. The U.S. herbal market

is growing tremendously with consumer demand way ahead of regulatory agencies. It is interesting to note that four (Ginkgo, St. John's Wort, Valerian, and Kava) of the top ten herbs purchased in the U.S. (according to 1999 Whole Foods Survey) have psychotropic activity. Recent trends in research into African plant uses show that traditional medicine is commonly used to treat neurological disorders in the West African region.

Natural products being easy to measure, cheap, and affordable have brought man closer to alternative therapy for the treatment of common diseases. In Nigeria, the use of these natural products in the treatment of mental and other illnesses should not be ignored (Akpanabiatu *et al*, 2006). *Rauwolfia vomitoria* (RV) is a natural product that has historically been used in Nigeria to treat psychiatric illnesses (Akpanabiatu *et al*, 2006).

Rauwolfia vomitoria is a member of the Apocynaceae family. It is mainly found in southern Nigeria's forest areas. The plant is also known as serpent wood in English as well as asofeyeje, ira, ira-igbo in Yoruba, wadda in Hausa, akata in Bini and Mmoneba and utoenyin in Efik (Orwa *et al*, 2009). *Rauwolfia vomitoria* is a shrub up to 8m in height (Orwa *et al*., 2009). According to (Akpanabiatu *et al*., 2006), the active phytochemical components are alkaloids, ajmaline serpentinine, saponin, rauwolfine, serpentine, steroid-serposterol, rescinnamine and reserpine, which is the active compound used in the treatment of psychosis. *Rauwolfia vomitoria* extracts have anti-inflammatory effect , antipyretic effect, anti-diabetic effect and anti-cancer effect (due to the β-carboline alkaloid, alstonine) (Umoh *et al*., 2006). *Rauwolfia vomitoria* has been reported to be relatively safe with a LD50 of 17.5 g/kg. *Rauwolfia vomitoria* extract has been very well characterised using a combination of the high-performance liquid chromatography (HPLC) and the high-performance thin-layer chromatography (HPTLC) on normal- and reverse-phase. Morealso, the indole akaloids with yohimbane skeleton namely yohimbine, reserpine, rescinnamine, raucaffricine, ajmaline and ajmalicine identified as the biologically active

substances in RV (Azeem *et al*., 2005). Similarly, RV leaves extract has been found to have high levels of zinc and vitamin C (ascorbic acid), both of which are essential for male fertility (Ogunlesi *et al*., 2009). RV crude extract has been reported to aid in the treatment of swellings in male reproductive organs associated with infertility (Sinclair, 2000). It has also been reported to have anti-prostate cancer and anti-diabetic properties, suggesting that it may be used as a fertility enhancer in cases where infertility is a problem (Sinclair, 2000).

Reserpine (RES) was first isolated in 1952 from the dried root of *Rauwolfia vomitoria* and had been used for centuries in India for the treatment of insanity, as well as fever and snakebites (Gomati *et al*., 2014). It was first used in the United States by Robert Wallace Wilkins in 1950 (Nicolaou & Sorensen, 1996) and it was introduced into the market in 1954, two years after Chlorpromazine. Nur & Adam, 2016 reported that reserpine irreversible pharmacology potency makes its better antipsychotic because its effect last longer than any other antipsychotic drugs. Though reserpine only as an antipsychotic drug has been reported to pose some side effects which make its use to be discontinued in the United Kingdom for some years but find its way back into the market by combining with other chemical such has chlorthalidone, thiazide e.t.c. (Nur & Adam, 2016).

Reserpine (methyl 18β-hydroxy-11, 17 α-dimethoxy-3β, 20α-yohimban-16β-carboxylate 3,4,5-trimethoxybenzoate), known by trade names Raudixin, Serpalan, Serpasil. Reserpine is an indole alkaloid, (Wayne's, 2005) antipsychotic, and antihypertensive drug that has been used for the control of high blood pressure and for the relief of psychotic symptoms. It is an antipsychotic drug, available as 0.1 mg and 0.25 mg tablets for oral administration.

Reserpine is a pure crystalline alkaloid of rauwolfia, is a White or pale buff to slightly yellowish crystalline powder with no odor. When exposed to light, it darkens slowly, but when in solution, it darkens more quickly. It is water insoluble but readily soluble in acetic acid, chloroform,

benzene, and in alcohol. Its molecular weight is 608.69. Reserpine depletes stores of catecholamine and 5-hydroxytryptamine in many organs, including the brain and adrenal medulla. Most of its pharmacological effects have been attributed to this action (usp.org).

1.1 Research Justification

Antipsychosis drugs have been shown to induce reproductive toxicity, and hypothalamic–pituitary–testicular-axis (HPTA) is responsible for the control of male reproductive functions (Xavier & William, 2010) and the mechanisms underlying HPTA damage as a result of antipsychotic drugs in most cases are poorly understood at the present time. There is a paucity of published studies on the subject in the reviewed literature. For ethical reasons it is difficult in humans to carry out studies that will follow the progress of morphological changes in the testes. This will for instance require serial biopsies of the testes to obtain tissue which may take a longer period of time to execute. It is more difficult in these situations to carry out controlled studies. This requires taking testicular tissue from fertile men. Animal studies with suitable models (Wistar rats) are therefore required.

Despite well-documented side effects and the introduction of a new generation of antipsychotic medications, chlorpromazine is still one of the most widely available and affordable therapies for schizophrenia (Norquist *et al*., 2009). Chlorpromazine is widely used in Africa (Odejide and Ban, 1982), while it is generally used in India, and the older generation of antipsychotics is used to treat the majority of people with schizophrenia in South East Asia (Chong *et al*., 2004). Chlorpromazine was the most commonly administered of the first generation 'typical' antipsychotic medications in the United Kingdom, where, at the time, the 'typical' category of antipsychotics, as well as its almost widespread usage in clinical practice, chlorpromazine serves as a guideline against which other treatments are measured (Adams *et al*., 2004). Thus Chlorpromazine was used for this study.

Natural products being easy to come by, cheap, and affordable have brought man closer to alternative therapy for the treatment of common diseases. In Nigeria, the use of these natural products in the treatment of mental and other illnesses should not be ignored (Akpanabiatu *et al.*, 2006). In Nigeria, *Rauwolfia vomitoria* is one of the traditional natural products used to treat psychiatric illnesses (Akpanabiatu *et al.*, 2006). RV leaves have been reported to contain high concentrations of reserpine which is the antipsychotic component. It leaves has also been reported to contain Zinc and Ascorbic acid (Vitamin C), the element which are essential to promote male fertility (Ogunlesi *et al.*, 2009). Thus, there is need to investigate the effect of *Rauwolfia vomitoria* leave extract and combined effect of reserpine, ascorbate and zinc (RAZ) all isolated from *Rauwolfia vomitoria* leaves on male reproduction.

1.2 Statement of the Research Problem

Synthetic antipsychosis drugs have been shown to induce reproductive toxicity, and hypothalamic–pituitary–testicular-axis (HPTA) is responsible for the control of male reproductive functions (Xavier & William, 2010). Moreover, traditionally used herb, *Rauwolfia vomitoria* has been reported to be potent antipsychotic agent that also promotes fertility, probably due to antioxidant presents in the plant.

Molecular mechanisms underlying HPTA damage as a result of synthetic antipsychotic drugs and how Rauwolfia vomitoria enhance fertility via HPTA axis are poorly understood at the moment. More also, no study has ever report the effects of co-administration of the potent phytochemicals (i.e. Reserpine, Ascorbate and Zinc (RAZ)) present in the Rauwolfia vomitoria on reproductive parameters.

1.3 Significant of the Study

The toxicity profiling of the compounds used (Chlorpromazine, Reserpine, Zinc, Ascorbic acid) would be of immense benefit to users and medical personnel. The findings of this study could

help to establish the safety or otherwise of the effects of Chlorpromazine, *Rauwolfia vomitoria* leave extract, Reserpine and combine administration of reserpine, ascorbate and zinc on male fertility.

Moreover, toxicity profiling of the pharmacodynamics synergy in RAZ may help to bring limelight to the development of new antipsychotic drugs that will be useful to psychotic patients without the fear of coming down with infertility problem. Furthermore, this may even encourage the practice of phytomedicine in Nigeria, has Nigeria is blessed with a lot of medicinal plant which scientist are yet to explore. Exploration of these medicinal plants can make Nigeria an independent country in herbal medicine and be the channel of source of income to Nigeria by exporting our products just like other developing countries like China, India, Malaysia and so on do.

1.4 Broad Objectives of the Study

This study aims to evaluate the comparative effects Chlorpromazine, *Rauwolfia vomitoria* leave extract and concurrent administration of RAZ isolated from Rauwolfia vomitoria leaves on the hypothalamic-pituitary-testicular-axis in adult Wistar rats.

1.5 The specific objectives

The specific objectives of this study are;

1. to determine the effect of Chlorpromazine, reserpine, Rauwolfia vomitoria leave extracts and co-administration of reserpine, zinc and vitamin C at different dosage on animal body weight gained,
2. to investigate expression of CAMP Responsive Element Modulator (CREM), protamine-1 (PRM-1) and protamine-2 (PRM-2) genes using reverse transcriptase-PCR techniques,
3. to determine the effect of Chlorpromazine, reserpine, Rauwolfia vomitoria leave extracts and co-administration of reserpine, zinc and vitamin C at different dosage on serum

levels of follicle stimulating hormone, luteinizing hormone, testosterone, prolactin and Gonadotropin releasing hormone (GnRH) using ELISA kit,

4. to investigate serum concentration of malondialdehyde, superoxide dismutase, glutathione peroxidase and dopamine using ELISA kits,
5. to investigate total sperm count, sperm morphology and motility after administration of Chlorpromazine, reserpine, Rauwolfia vomitoria leave extracts and co-administration of reserpine, zinc and vitamin C at different dosage,
6. to determine histoarchitecture of the testes and hypothalamus after administration of Chlorpromazine, reserpine, Rauwolfia vomitoria leave extracts and co-administration of reserpine, zinc and vitamin C at different dosage using Hematoxylin and Eosin technique,
7. to determine expression of cytokeratin, Bcl2 and Ki-67 proteins in the testes of the animals' using immunohistochemical technique,
8. to quantify germinal cell count using physical fractionators' techniques.

1.6 Limitation of the Study

The study was limited to the use of male Wistar rat in creating a model for examining the level of infertility associated with antipsychotic drugs.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 The Hypothalamic-Pituitary-Gonadal Axis

The reproductive axis must be understood in order to diagnose irregular genital development (e.g. pseudo hermaphroditism), hypergonadism, hypogonadism, infertility, and erectile dysfunction (Jeffrey, 2010). The hypothalamus, pituitary gland, and testis are the three key components of the reproductive hormonal axis in males. This axis' regulation has an effect on steroid-sensitive end organs like the prostate and penis (Jeffrey, 2010). HPTA produces the concentrations of circulating steroids necessary for normal male sexual growth, sexual function, and fertility in a tightly controlled manner (Jeffrey, 2010).

The hypothalamus is the integrating nucleus of the reproductive hormonal axis. The peptide hormone gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus and transported to the adenohypophysis of the pituitary gland through a short portal venous system, where it activates the synthesis and release of gonadotropic hormones (luteinizing hormone-LH and follicle stimulating hormone-FSH). Both neural input from the central nervous system and humoral factors from the testis modulate the secretion of GnRH (Tony, 2015). The pulsatile pattern of GnRH release appears to be essential for stimulating LH and FSH release, while constant exposure to GnRH has paradoxical inhibitory effects on LH and FSH release. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are glycopeptides consisting of two peptide chains (alpha and beta). Although named after their function in females, they are produced by both sexes, secreted into the general circulation and thereby transported to the testis. LH and FSH are synthesized in the pituitary gland, released into the systemic blood circulation, and carried to the target end organs the gonads (Tony, 2015).

In the testis, LH stimulates testosterone secretion and FSH is important in the initiation and maintenance of spermatogenesis. The secreted testicular androgen testosterone and its activated form dihydrotestosterone (DHT) act on numerous target end organs causing the development of male secondary sexual characteristics and inhibiting the pituitary secretion of LH and FSH. Peptide secretory products of the testis include inhibin, activin and follistatin which also regulate gonadotropin secretion. Sertoli cell products may serve as the mediators of interaction between germ cells, Leydig cells, peritubular myoid cells and the Sertoli cells of the testis. The development of the male germ cells in the seminiferous tubule essentially consists of three phases: spermatogonal clonal expansion, meiosis, and spermatogenesis. LH stimulates testicular steroidgenesis by binding to receptor on leydig cells. In addition to LH, FSH may directly affect leydig cells function by action on the sertoli cells and spermatogenesis (Tony, 2015).

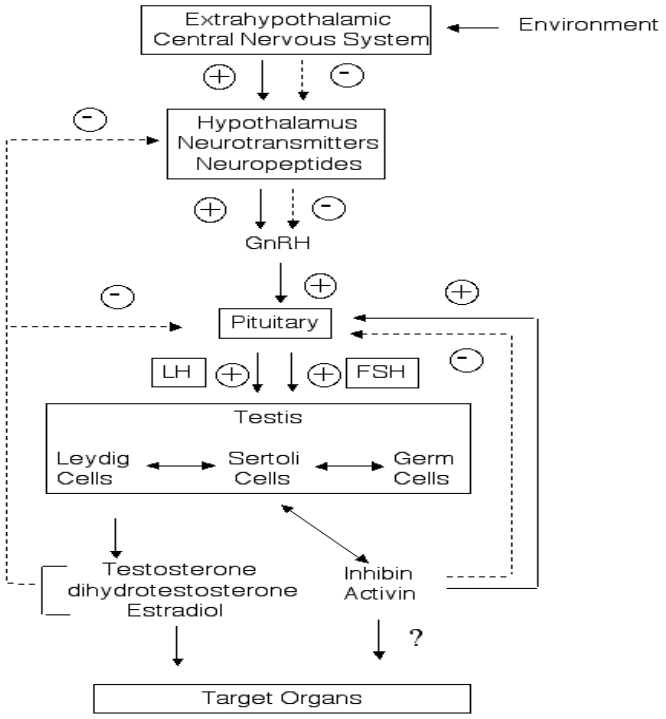


Figure 2.1: Shown schematic illustration of HPG-axis regulation (Adapted from Campbell's Urology, 8th Edition)

2.1.1 The Hypothalamus

The hypothalamus extend from the optic chiasma to the caudal boundary of the mammillary bodies and its a member of the diencephalon. It is located underneath the thalamus and forms the floor of the third ventricle as well as the inferior part of the lateral walls. A region called the preoptic area (POA) is located anterior to the hypothalamus and physiologically, it's often included in the hypothalamus. The hypothalamus is made up of nerve cells grouped in groups or nuclei, several of which are not clearly separated from one another under the microscope. These nuclei are divided into three zones for the purposes of description: periventricular, lateral and medial (Saper, 2004; Snell, 2010). The arcuate (Arc) and the anteroventral periventricular (AVPV) nuclei are the major sites in the hypothalamus that are located in its periventricular zone and involved in the control of reproduction (Dhillo, 2008). There is a general believe that estrogen with positive feedback effects on AVPV neurons, causes pre-ovulatory surges of gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) (Simerly, 2002). Destruction of the AVPV, but not neighboring nuclei, were found to result in persistent estrous and the abolition of the estrogen-induced LH surge (Wiegand *et al*., 1980). Unlike AVPV nucleus that involved in GnRH surge, it has been reported that GnRH pulse generator has been localized to or near the Arc in rat (Ohkura *et al*., 1991). Kisspeptin expressing neurons that have potent stimulatory effect on GnRH cells are mainly located in the Arc and AVPV nuclei of rat hypothalamus (Clarkson & Herbison, 2006).

The hypothalamus is the integrative core of the reproductive axis, receiving neuronal feedback from a variety of brain regions and acting as a pulse generator for pituitary and gonadal hormone secretion (Kleiman *et al*., 2001). The hypophyseal portal microvascular system is responsible for the pituitary gland's role and anatomical relationship with the hypothalamus. The portal vascular system allows hypothalamic-releasing hormones to be delivered directly to the anterior pituitary

gland. Reverse flow through this vascular path, on the other hand, can give pituitary hormones direct access to the hypothalamus. The pituitary gland is stimulated by the single hypothalamic decapeptide GnRH, resulting in the synthesis and release of both gonadotropic hormones (LH and FSH) (Kleiman *et al*., 2001). GnRH is released into the portal circulation in pulses that occur every 70 to 90 minutes on average, and it has a very short half-life in the blood of around 2 to 5 minutes. The pulsatile secretion of GnRH is necessary for LH and FSH stimulation and release. Alternately; continuous exposure of the gonadotropins to GnRH results in paradoxical inhibitory effects on LH and FSH, and the pituitary becomes desensitized by the downregulation of pituitary receptors (Silber, 2000). GnRH secretion may be affected by a number of factors, including stress, diet, exercise and age. The hypothalamus is controlled by neurotransmitters (serotonin, acetylcholine, dopamine, and norepinephrine) as well as neuropeptides (endogenous opioid peptides). The frequency and amplitude of GnRH secretion are also influenced by pituitary gonadotropins and gonadal steroids (Silber, 2000).

2.1.2 The Pituitary Gland

The pituitary gland (hypophysis) is a small gland that lies in the sella turcica, a bony cavity at the base of the brain, and is connected to the hypothalamus by the pituitary (or hypophysial) stalk. It is around 1 centimeter in diameter and weighs 0.5 to 1 gram. The anterior pituitary, also known as the adenohypophysis, and the posterior pituitary, also known as the neurohypophysis, are the two sections of the pituitary gland.

Ratheke's pouch is an embryonic invagination of the pharyngeal epithelium that gives rise to the anterior pituitary gland. A neural tissue outgrowth from the hypothalamus gives rise to the posterior pituitary.

Six important peptide hormones plus several hormones of lesser importance are secreted by the anterior pituitary, and two important peptides are secreted by the posterior pituitary. The

hormones secreted by the anterior pituitary include: Growth hormone, Adrenocorticotropin, Thyroid- stimulating hormone, prolactin and the gonadotropic hormones (follicle stimulating hormone and luteinizing hormone). While the hormones secreted by the posterior pituitary are Antidiuretic hormone and Oxytocin (Swerdloff *et al*., 2011)

2.1.2.1 Anterior Pituitary

GnRH influences the release of the two primary pituitary hormones (LH and FSH) that control testicular function by binding to high-affinity receptors on pituitary cells (gonadotrophs). Both of these pituitary hormones are glycoproteins with two polypeptide-chain subunits each. The protein component of α -subunits is identical and similar to that of other pituitary hormones (thyroid-stimulating hormone [TSH] and human chorionic gonadotropin [HCG]), but the unique - β-subunit confers biological and immunological activity.

The longer half-life of FSH in circulation is expressed by a more stable serum level than that of the more rapidly metabolized LH, despite the fact that both hormones are secreted episodically by the pituitary gland. Since a single measurement of circulating LH may be as much as 50% above or below the mean integrated hormone concentrations, the peak and trough pattern of blood levels of gonadotropins, especially LH, is clinically significant (Swerdloff *et al*, 2011).

2.1.3 Feedback Control of Gonadotropins

Testosterone exerts negative feedback on GnRH release through androgen receptors in hypothalamic neurons and the pituitary gland. This is easily demonstrated by the rise in serum LH and serum FSH that occurs after orchiectomy. LH and FSH blood levels continue to rise for a long period after castration, reaching maximum levels as late as 25 to 50 days after surgery. Estradiol, a potent estrogen, is produced both from the testis and from peripheral conversion of androgens and androgen precursors and is the predominant regulator of FSH secretion in the male. Estrogens provide input to the pituitary to modulate the gonadotropin secretion response to

each GnRH surge, while testosterone acts primarily as a feedback at the hypothalamic stage. Inhibin, a peptide growth factor produced by seminiferous tubules, is also important in the feedback regulation of pituitary FSH. Inhibin has also been isolated and characterized in follicular fluid. Two forms of inhibin have been isolated. They have the same alpha subunit, but their beta subunits are different. Inhibin B (alpha subunit and B variant of the beta subunit) is the form secreted by the Sertoli cells. By inhibiting transcription of the gene encoding the beta subunit of FSH, Inhibin B selectively suppresses FSH secretion in gonadotropes (Nelson, 2013).

2.2 Anatomy of the Testes

Embryologically, mammalian testes develop from the gonads. At first, the gonads can develop into either ovaries or testes (Scott *et al*., 2000). The gonadal rudiments are present in the intermediate mesoderm adjacent to the developing kidneys in humans beginning around week four. Inside the developing testes, sex cords begin to form around week 6. (Scott *et al*., 2000). Early Sertoli cells surround and nurture germ cells as they migrate into the gonads shortly before sex determination starts. In males, the Y-chromosome-encoded sex-specific gene SRY initiates sex determination through downstream regulation of sex-determining factors (GATA4, SOX9, and AMH), resulting in the formation of the male phenotype, which includes directing development of the early bipotential gonad down the male path of development (Scott *et al*., 2000).

The testes descend from the posterior fetal abdomen to the inguinal ring, then to the inguinal canal and into the scrotum along the "axis of descent." In most cases (97 percent full-term, 70 percent preterm), both testes have descended by birth. In most other instances, only one testis fails to descend (cryptorchidism) and it will possibly manifest itself within a year (Scott *et al*., 2000).

The testicle is otherwise called testes, (from Latin testiculus, diminutive of testis, meaning "witness" of virility). Testes is the male generative gland in animal (The American heritage dictionary, 4th edition). Like the ovaries to which they are homologous, testes are components of both the reproductive system (Gonads) and the endocrine system (Endocrine glands). The respective functions of the testes are sperm production (Spermatozoa) and sex hormone production (Reddy, 1996).

The testes are a pair of organ in the scrotum, measured 4 x 3 x 2.5 cm and 20-25ml in volume. Surrounding the testicles is a strong capsule called tunica albuginea testis. The testicular parenchyma is made up of 250-350 lobules that drain to the epididymis through the mediastinum testis. Connective tissue septa (septula testis) that originate in the mediastinum testis isolate the lobules. A testis lobule is made up of one or more seminiferous tubules that end and begin at the rete testis (Benninghoff, 1993).

From outside to inside, the spermatic cord is encased by the scrotal skin, tunica dartos, external spermatic fascia, cremasteric muscle, and internal spermatic fasci (Benninghoff, 1993).

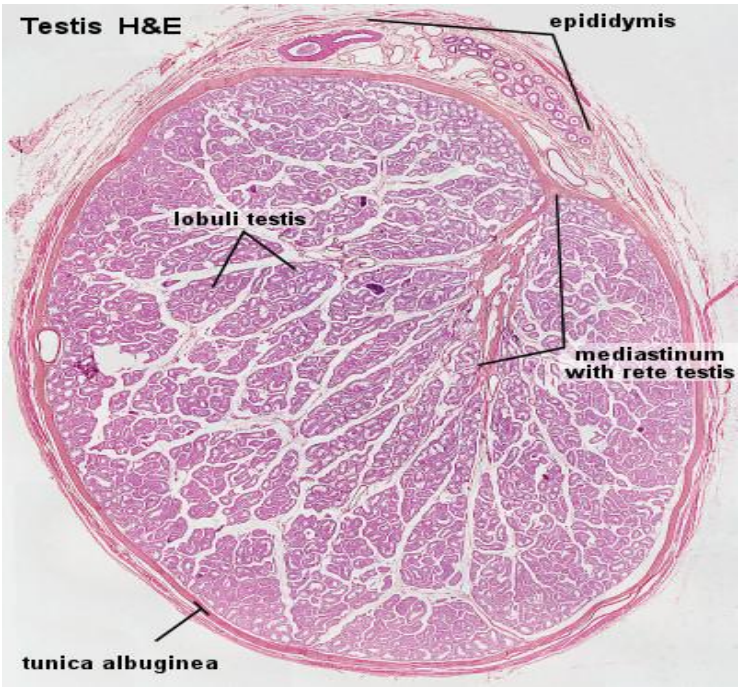


Figure 2.2: Shown Cross section of the testis and epididymis (Lutz, 2009).

2.2.1 The Convoluted Seminiferous Tubules

A dense basal lamina surrounds these tubules, which are surrounded by 3-4 layers of smooth muscle cells (or myoid cells). Seminiferous epithelium lines the insides of tubules, and it is made up of two groups of cells: spermatogenic cells and Sertoli cells (Lutz, 2009).

2.2.2 Spermatozoa

Matured, long and actively motile spermatozoon is usually 60 µm in human. Head, neck, and tail are three major parts of spermatozoon. The nucleus is the main component of the head which contained condensed chromatin, spermatozoon head is flat, 5 µm long and 3 µm wide). The acrosome covers the anterior two-third of the nucleus contain an enzymes which is essential in the fertilization process while the basal plate is formed by the posterior sections of the nuclear membrane (Lutz, 2009).

The neck is attached to the basal plate and is short (about 1 µm). Immediately behind the basal plate is a transversely oriented centriole. There are nine segmented columns of fibrous material in the spermatozoon neck and these proceed as thick outer fibers into the tail (Lutz, 2009).

The tail is further divided into three pieces: a middle piece, a principal piece and an end piece. In the middle piece, the axonema (the common term for the arrangement of microtubules in all cilia) begins. It is covered by nine thick outer fibres that are absent from other cilia.

In the middle piece (about 5 µm long), the axonema and dense fibres are surrounded by a sheath of mitochondria. The middle piece is terminated by a dense ring, the annulus. The principal piece is about 45 µm long. It contains a fibrous sheath, which consists of dorsal and ventral longitudinal columns interconnected by regularly spaced circumferential hoops. The fibrous sheath and the dense fibres do not extend to the tip of the tail. Along the last part (5 µm) of the tail, called the end piece, the axonema is only surrounded by a small amount of cytoplasm and the plasma membrane (Lutz, 2009).

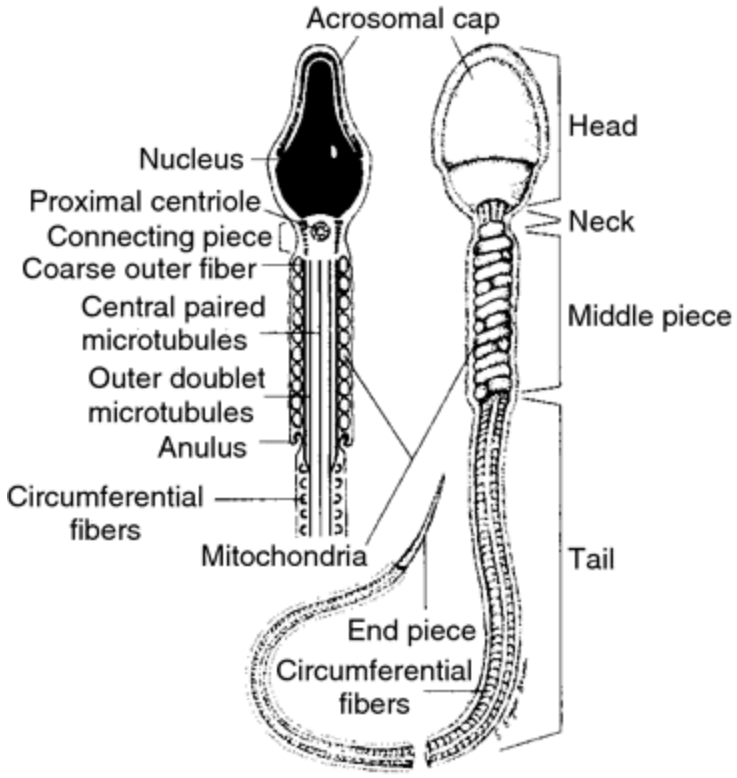


Figure 2.3: Shown schematic anatomy of the mature spermatozoa (Lutz, 2009).

It takes about 48 days from the time cells enter meiosis until morphologically mature spermatozoa are formed. Depending on the length of reproduction of spermatogonia (which is not precisely determined) it takes approximately 64 days to complete spermatogenesis in human and 56 days in rats. Spermatogenesis is regulated by follicle stimulating hormone (FSH), which in males stimulates the spermatogenic epithelium, and luteinizing-hormone (LH), which in males stimulates testosterone production by Leydig cells in the interstitial tissue (Lutz, 2009).

2.2.3 Sertoli Cells

Sertoli cells are far less numerous than the spermatogenic cells and are evenly distributed between them. Their shape is highly irregular - columnar is the best approximation. Sertoli cells extend from the basement membrane to the luminal surface of the seminiferous epithelium. Processes of the Sertoli cells extend in between the spermatogenic cells (Lutz, 2009). Sertoli cells have an ovoid nucleus that is big and lightly stained, and also has a large nucleolus. The

long axis of the nucleus is perpendicular to the tubule's wall. Sertoli cells' lateral processes are bound by close junctions, which are thought to be the structural foundation for the blood-testis barrier. The basal compartment contains spermatogonia and primary spermatocytes, while the adluminal compartment contains other cellular stages of spermatogenesis. Tight junctions can open temporarily to allow spermatogenic cells to move from the basal to the adluminal compartment. Sertoli cells assist spermatogenic cells mechanically and nutritionally. Inhibin and activin are two hormones secreted by Sertoli cells that provide positive and negative feedback on pituitary FSH secretion (Lutz, 2009).

2.2.4 Vascular Supply of the Testes

The testicular artery (from the aorta), the deferential artery (from the internal iliac artery), and the Cremasteric artery (from the inferior epigastric artery) are the arteries of the spermatic cord. Pampiniform plexus (Venous plexus in the spermatic cord), which forms the testicular vein and empties into the renal (left side) or vena cava are spermatic cord veins (right side). The lymphatic system of the testes drains to the para aortic lymph nodes through the spermatic cord. The lymphatic vessels of the scrotum drain to the lymph nodes in the inguinal region. The testes' autonomic nerve supply comes from the para aortic ganglia (main function is the sympathetic innervations of the testicular vessels). The genitofemoral nerve (which originates in the lumber plexus L1-L2) passes through the inguinal canal and gives rise to the ramus genitalia (It supplies the scrotal skin, cremasteric muscle and tunica dartos). The ilioinguinal nerve (which originates in the lumber plexus L1) supplies the skin of the scrotum, the groin, and the medial thigh (Benninghoff 1993).

2.2.5 Spermatogenesis

The systematic transition of germ cells into spermatozoa is known as spermatogenesis. It occurs primarily in the testes' seminiferous tubules and is divided into three stages, each of which is associated with a different type of germ cell (Ellen and Herman, 2017). The proliferative phase include spermatogonia to spermatocytes, the meiotic phase involve conversion of spermatocytes into spermatids and the differentiation phase also known as spermiogenesis involve transformation of spermatids into spermatozoa (Ellen and Herman, 2017).

Spermatogenesis takes place in the seminiferous tubules. The germ cells, which will grow into sperm, and somatic cells known as sertoli cells, which nurture the germ cells during the development process, are the two main cell types involved in spermatogenesis. The stem cells migrate slowly from the basement membrane of the tubules through the close junctions between the sertoli cells into the tubular lumen as they pass through their stages of growth (Ellen and Herman, 2017).

2.2.5.1 Stages of spermatogenesis

Proliferative phase: Mitosis occurs in stem cells in the tubular epithelium's basal zone. These divisions' offspring retain their own numbers while also producing many interconnected B spermatogonia. Primary spermatocytes are produced by B spermatogonia. All descendants of B spermatogonium are connected by cytoplasmic bridges, forming a syncytium-like clone that develops in lockstep (Ellen and Herman, 2017).

Meiotic phase: Primary spermatocyte divides into two short-lived secondary spermatocytes, each of which produces two spermatids. The number of chromosomes in spermatids is haploid. The number of chromosomes in spermatids is haploid. The largest cells in the spermatogenic series is primary spermatocytes which are found in the middle of the seminiferous epithelium.

Meiosis is a lengthy procedure, with the prophase of the first meiotic division taking up to three weeks (Ellen and Herman, 2017).

Differentiation phase (Spermiogenesis): Here spermatids become spermatozoa after undergoing transformation. The formation of the acrosome, which covers the cranial portion of the spermatozoon head, is one of the three main changes that occurs within the cells. Hydrolytic enzymes in the acrosome cause sperm and egg to fuse for fertilization, the condensation of nuclear chromatin in the head to form a dark-staining structure, the growth of the tail opposite the acrosome is the second change and the loss of excess cytoplasmic material that is shed as a residual body is the last changes that occurs in this phase. The residual body is phagocytosed by the sertoli cells (Ellen and Herman, 2017).

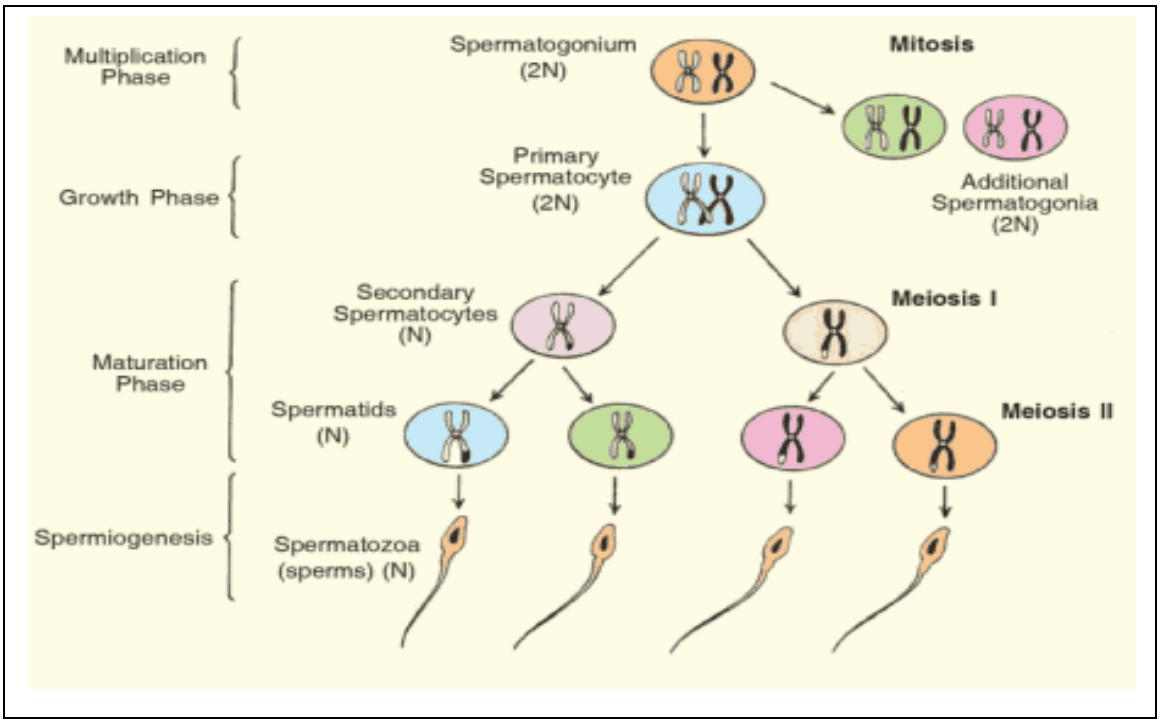


Figure 2.4: showing the stages of spermatogenesis (encyclopedia 2015)

2.3 Ascorbic Acid

Vitamin C also known as ascorbic acid or ascorbate is a water-soluble micronutrient that is important for a number of biological processes. Ascorbic acid is a cofactor for enzymes involved in collagen hydroxylation after translation, carnitine biosynthesis, and dopamine conversion to norepinephrine, peptide amidation, and tyrosine metabolism. In addition, vitamin C controls iron absorption by transforming ferric Fe^{3+} to ferrous Fe^{2+} ions in the gastrointestinal tract, encouraging dietary non-haem iron absorption and stabilizing iron-binding proteins (Aysun, 2009).

While most animals can synthesize vitamin C from glucose, humans, other primates, guinea pigs, and fruit bats lack the last enzyme needed for vitamin C synthesis (gulonolactone oxidase) and therefore need vitamin C in their diet. As a consequence, long-term vitamin C deficiency causes defects in collagen post-translational alteration, leading to scurvy and death. Vitamin C is a powerful reducing agent and scavenger of free radicals in biological systems, in addition to its antiscorbutic action (Aysun, 2009).

In living organisms, ascorbic acid is involved in a number of physiological functions. It is well known for its role in the synthesis of collagen in connective tissues (Aguirre and May, 2008). Scurvy is characterized by the absence of wound healing and the failure of fractures to heal. These characteristics are due to a lack of vitamin C, which causes impaired collagen production. Ascorbic acid is a potent reducing agent that oxidizes to dehydroascorbic acid in a reversible manner.

According to research on the interactions of ascorbic acid with various chemicals and metal ions, ascorbic acid and its oxidation product dehydroascorbic acid, as well as its intermediate monodehydroascorbic acid free radical, can serve as cycling redox couples in electron transport and membrane electrochemical potentiation reactions (Packer and Fuchs, 1997). Numerous

biochemical experiments have been performed on electron transport and redox coupling reactions. Ascorbic acid, for example, has been shown to play a role in a number of neurochemical reactions involving electron transport.

Ascorbic acid is used by neurons in a number of chemical and enzymatic reactions, including the synthesis of neurotransmitters and hormones (Packer and Fuchs, 1997). Extracellular ascorbic acid interactions with various plasma membrane proteins indicate that ascorbic acid can act as a neuromodulator (Packer and Fuchs, 1997).

The role of ascorbic acid in cellular metabolism can be explained by its reducing properties, which protect cellular components from oxidative damage, among other things. It scavenges oxidizing free radicals and dangerous oxygen-derived species like the hydroxyl radical, hydrogen peroxide, and singlet oxygen (Arrigoni and De Tulio, 2002).

The prooxidant activity of ascorbic acid is known to stimulate some biochemical reactions. Ascorbic acid's bactericidal and antiviral activity in aqueous solution is thought to be due to its prooxidant properties (Arrigoni and De Tulio, 2002).

2.3.1 Molecular Structure

L-ascorbic acid is a dibasic acid with a five-membered heterocyclic lactone ring and an enediol group. Ascorbic acid's chemical and physical properties are linked to its structure (Aguirre and May, 2008). Dehydroascorbic acid (DHA), the first oxidation result of ascorbic acid (Fig. 2.5), has been determined to be a dimer by x-ray crystallography.

According to electrochemical studies, ascorbic acid and dehydroascorbic acid form a reversible redox couple. The molecule of ascorbic acid is made up of two asymmetric carbon atoms, C-4 and C-5. As a result, there are three other stereoisomers of L-ascorbic acid: D-ascorbic acid, D-isoascorbic acid, and L-isoascorbic acid. The antiscorbutic activity of these three isomers is either negligible or non-existent (Coassin *et al*., 1991).

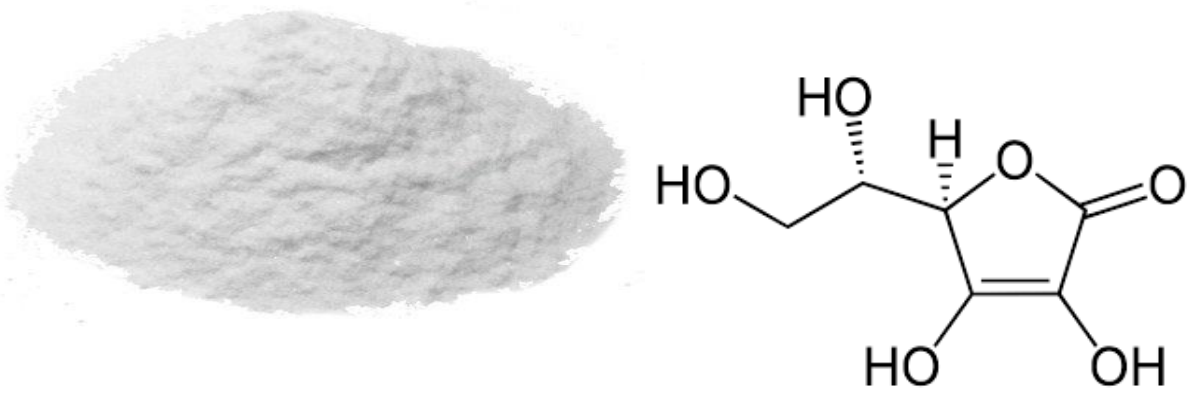


Figure 2.5 shown image and chemical structure of dehydroascorbic acid (Caspi, 2000).

2.3.2 Biosynthesis of L-ascorbic acid

The glucuronic acid metabolic pathway involves the biosynthesis of ascorbic acid in animals. The metabolic pathway is involved in both normal and disease sugar metabolism, as well as the regulation of physiological functions. It's a crucial route for big detoxification processes. The activities of the synthesizing enzymes differ depending on the species (Aguirre and May, 2008). D-glucose can be converted to L-ascorbic acid by almost all animals. Endogenous ascorbic acid development is not possible in humans, other primates, guinea pigs, Indian fruit bats, some fish and birds, and insects. Rats have been used in the majority of studies on ascorbic acid synthesis in animals.

D-glucuronic acid, L-gulonic acid, L-gulonolactone, and 2-keto-L-gulonolactone function as intermediates in the conversion of D-glucose to L-ascorbic acid. The inversion of C-1 and C-6 between Dglucuronic acid and L-gulonic acid occurs in the synthetic pathway, according to studies using radioactive labeling methods, while the D-glucose chain remains intact.

The oxidizing enzyme L gulono—lactone oxidase is absent in animals that cannot synthesize ascorbic acid endogenously. This enzyme is necessary in the final step of the conversion of L-gulono-lactone to 2-oxo-L-gulono-lactone, a tautomer of L-ascorbic acid that spontaneously transforms into vitamin C. (Nishikimi *et al*., 1994).

Ascorbic acid or one of its isomers may be synthesized by certain microorganisms. L-gulono—lactone dehydrogenase, a bacterial-origin enzyme that catalyzes the oxidation reaction of ascorbic acid synthesis, has been isolated and characterized. This enzyme has completely different physical and chemical properties than eucaryotic organisms.

2.3.3 Metabolism of L-ascorbic acid

In a sequence of reactions, ascorbic acid is metabolized in the liver and, to a lesser degree, the kidney. The main mechanism for the synthesis of ascorbic acid requires the loss of two electrons. The intermediate free radical forms dehydroascorbic acid reversibly, resulting in the formation of the physiologically inactive 2,3-diketogulonic acid, which is irreversible. Diketogulonic acid can be cleaved to form oxalic and threonic acids, or it can be decarboxylated to form carbon dioxide, xylose, and xylulose, which leads to xylonic acid and lyxonic acid. Many of these metabolites, as well as ascorbic acid, are excreted in urine.

2.3.4 Ascorbic acid redox metabolism

Vitamin C is an effective reducing agent and scavenger of free radicals in biological systems, in addition to its antiscorbutic action (Duarte and Lunec, 2005). At physiological pH, the mono-anion type (ascorbate) is the most common chemical species. Ascorbate easily undergoes two one-electron oxidations in a row, both of which are reversible, to produce dehydroascorbate (DHA) and an intermediate, the ascorbate free radical (AFR). However, compared to the -tocopherol radical, glutathione radical, and nearly all reactive oxygen and nitrogen species (e.g. hydroperoxyl radicals, superoxide anion, nitrogen dioxide, hydroxyl radical, hypochlorous acid, singlet oxygen and nitroxide radicals) thought to be implicated in human disease, AFR is a relatively unreactive free radical with a low reduction ability (Duarte and Lunec, 2005).

2.3.5 Ascorbic acid availability and transport

Ascorbic acid is a water-soluble vitamin that is readily absorbed by the alimentary system. Good, well-nourished, non-smoking people have plasma ascorbic acid levels of 50-60 μM. (Rose *et al*., 1993; Buettner and Jurkiewicz, 1996). Plasma levels can be raised by a long-term vegetarian diet (Woollard *et al*., 2002) as well as oral supplementation up to 100 μM. (Brennan *et al*., 2002). Due to efficient vitamin C excretion in the urine, higher plasma levels are not observed even with supplementary doses greater than 500 mg/day (Levine *et al*., 1996).

According to some research, an increase in plasma vitamin C levels is followed by an increase in intracellular vitamin C levels; however, this increase is also not dosedependent, possibly due to cellular saturation. Thus, it is understood that neutrophils, monocytes, and lymphocytes' intracellular vitamin C concentrations saturate at lower supplementation doses than human plasma.

In vitro studies have shown that cellular vitamin C transport is mediated by two distinct mechanisms. The sodium electrochemical gradient drives ascorbate into mammalian cells through a family of unique transporters. Vitamin C is absorbed primarily in the lower part of the intestine through active transport and via the sodium-dependent vitamin C transporter-type 1 ascorbate transporter (SVCT1, gene product of slc23a1). This transporter is also located in the proximal tubules of the kidney, where it is responsible for reabsorption of filtered ascorbate. The concentration of ascorbate in the blood is 30-60 μM, but it is much higher in most cells. This is due to active transport by the SVCT2 isoform of the ascorbate transporter (gene product of slc23a2). SVCT2 is found in most body tissues, including the brain, lung, liver, cardiac, and skeletal muscle. Because of saturation of absorption, uptake into tissues and failure of full reabsorption in the kidney, plasma concentrations of ascorbate are restricted to around 120 μM (Tsukaguchi *et al*., 1999). Notably, through facilitated diffusion through many isoforms of

the glucose transporter (GLUT), the oxidized form DHA is transported into cells faster than the reduced form (Vera *et al*., 1993), a mechanism that can be disrupted by glucose in some but not all cell types.

2.3.6 Biological pathways of L-ascorbic acid

Free radicals are formed in the body as a result of biological processes and in response to exogenous stimuli, and they are regulated by a variety of enzymes and antioxidants. When the production of free radicals outnumbers the body's capacity to neutralize them, oxidative stress occurs, which can lead to tissue damage following trauma, inflammatory events, and chronic diseases like atherosclerosis, degenerative disease, and cancer (Ames *et al*., 1994). Antioxidant vitamins such as vitamin E, vitamin C, and carotene have been shown to reduce oxidative damage in humans, reducing the risk of certain chronic diseases. Low plasma concentrations of L-ascorbic acid, tocopherol, and carotene have been linked to cardiovascular disease in epidemiological studies.

Ascorbic acid aids in the formation of hydroxyproline, hydroxylysine, norepinephrine, serotonin, homogenistic acid, and carnitine by assisting in the metabolism of several amino acids. Collagens, the fibrous connective tissue in animals, contain hydroxyproline and hydroxylysine. Tendons, ligaments, skin, bone, teeth, cartilage, heart valves, intervertebral disks, cornea, eye lens, and the ground substances between cells all contain collagens. Proline and lysine are hydroxylated posttranslationally on the growing polypeptide chain when collagen is synthesized. For the formation of a stable extracellular matrix and cross-links in the fibers, hydroxyproline and hydroxylysine are necessary. Only by hydroxylating the necessary proline and lysine residues can the triple helix quaternary state of physiologically effective collagen be achieved. Ascorbic acid deficiency decreases the activity of two mixedfunction oxidases that hydroxylate proline and lysine, prolylhydroxylase and lysyl hydroxylase. Ascorbic acid's function is most

likely to hold the iron cofactor in a reduced state at the active sites of the hydroxylases. In the absence of ascorbic acid, some collagen forms, but the fibers are irregular, resulting in skin lesions and blood vessel fragility, which are scurvy symptoms (Diliberto and Daniels, 1991).

Cell growth and differentiation may also be affected by L-ascorbic acid. Depending on the cell type, L-ascorbic acid inhibits or promotes tumor cell formation. The inhibitory effect of L-ascorbic acid is not limited to the biologically active isomer, and isoascorbate and D-ascorbic acid are more effective at inhibiting cell growth than L-ascorbic acid (Alcain and Buron, 1994). In human arterial endothelial cells, L-ascorbic acid and α-tocopherol, alone or in combination, induced proliferation and DNA synthesis, as well as antagonized the anti-proliferative effects of oxLDL, while VSMC proliferation was inhibited (Ulrich-Merzenich *et al*., 2002). Thus, L-ascorbic acid and -tocopherol can act as "preventative" agents against atherosclerotic plaque formation by promoting re-endothelization and then inhibiting VSMC proliferation (Ivanov *et al*., 1997).

Ascorbic acid regulates and participates in neurotransmitter enzymatic reactions and transport, as well as hormone biosynthesis. Ascorbic acid is involved in many hydroxylation and decarboxylation reactions in the biosynthesis of a number of neurochemicals. Normally, homogenistic acid catabolizes tyrosine to fumaric and acetoacetic acid (Diliberto and Daniels, 1991).

Tyrosine synthesis is incomplete in animals deficient in ascorbic acid. Tyrosine is metabolized to catecholamines through hydroxylation and decarboxylation in the presence of ascorbic acid, resulting in dopamine, norepinephrine, epinephrine, and adrenocrome. Ascorbic acid plays a direct role in the formation of norepinephrine by the dopamine-hydroxylase reaction. The primary result of oxidation may be the ascorbate free radical. Catecholamine biosynthesis takes

place in the adrenal glands and the brain, all of which use a lot of ascorbic acid (Arrigoni and De Tulio, 2002).

Ascorbic acid protects catecholamines by direct chemical interactions and the elimination of adrenocrome, a harmful byproduct of catecholamine oxidation related to some mental illnesses (Arrigoni and De Tulio, 2002). The use of the folic acid derivative tetrahydrobiopterin as an electron carrier and the recycling of ascorbic acid seem to be involved in the hydroxylation of tyrosine to catecholamines and the hydroxylation of phenylalanine to tyrosine. Oxidized dihydrobiopterin as a substrate can be restored by ascorbic acid. Dopamine-hydroxylase is thought to recycle tetrahydrobiopterin in combination with monodehydroascorbate reductase (Eipper and Mains, 1991).

The hydroxylation and decarboxylation of tryptophan is needed for the synthesis of serotonin, a neurotransmitter and vasoconstrictor. Ascorbic acid is thought to be required for the first hydroxylation step, which is catalyzed by tryptophan hydroxylase. Tetrahydrobiopterin is the hydroxylase's cosubstrate. It's been proposed that ascorbic acid will recover this substrate from its dihydrobiopterin oxidized state (Eipper and Mains, 1991).

Carnitine can be present in the heart and skeletal muscles, as well as the liver and other body tissues. It is needed for the transport of energy-dense activated long-chain fatty acids from the cytoplasm to the matrix side of the mitochondrial membrane, where they are catabolized to acetate.

Two hydroxylases convert lysine and methionine into carnitine in a series of reactions that involve ferrous iron and ascorbic acid to complete. Vitamin C deficiency slows carnitine biosynthesis, reduces renal carnitine reabsorption efficiency, and increases urinary carnitine excretion; these effects can explain the accumulation of triglycerides in the blood, as well as the physical weakness and lassitude associated with scurvy (Reboucne, 1995). The hormones

oxytocin, vasopressin, cholecystokinin, and -melanotropin improve their stability and maximum activity when they are amidated by L-ascorbic acid. L-ascorbic acid increases iron absorption by reducing Fe3+ to Fe2+ from non-heme iron sources. In vitro, L-ascorbic acid acts as a pro-oxidant in the presence of redox-active iron and may contribute to the development of hydroxyl radicals, which may eventually lead to lipid, DNA, or protein oxidation (Teucher *et al*., 2004). However, there was no pro-oxidant effect on L-ascorbic acid supplementation when DNA damage was assessed in the presence or absence of iron. Phosphatase calcineurin protein is inactivated by superoxide anion and L-ascorbic acid protect and reactivate it, thus this suggests that reduced iron is needed for phosphatase activity, implying that vitamin C can modulate signaling pathways in the cardiovascular system through calcineurin activation.

L-ascorbic acid's chemical and biological properties suggest that it can act as an antioxidant in vivo (Rose and Bode, 1993). The production of atherosclerosis is related to lipid peroxidation and oxidative modification of low density lipoproteins (LDL). L-ascorbic acid prevents isolated LDL from oxidation by various forms of oxidative stress by scavenging reactive oxygen species. LDL oxidation is prevented in an in-vitro study where L-ascorbic acid worked as a synergistic antioxidant with α-tocopherol. Furthermore, in vitro studies have shown that physiological concentrations of L-ascorbic acid inhibit LDL oxidation by vascular endothelial cells significantly. The adhesion of leucocytes to the endothelium is an essential step in the initiation of atherosclerosis; in vivo, L-ascorbic acid inhibits leucocyte-endothelial cell interactions caused by cigarette smoke or oxidized LDL.

By inactivating nitric oxide (NO), which regulates arterial tone and inhibits local inflammation, coagulation, and cell proliferation, superoxide and oxLDL can cause endothelial dysfunction (Tousoulis *et al*., 1999). Endothelial activity and vasodilation are harmed by oxidative stress and oxLDL, which decrease nitric oxide bioavailability in the artery wall, events that can be avoided

by L-ascorbic acid. Coronary and brachial endothelial vasomotor activity did not change over six months in a long-term study with vitamin C and E supplementation. In this study, vitamins C and E reduced F2-isoprostanes but had no effect on oxLDL formation or autoantibodies to oxLDL. Long-term oral vitamin C and E supplementation did not boost key pathways in atherosclerosis biology or endothelial dysfunction, nor did it minimize LDL oxidation in vivo (Kinlay *et al*., 2004). Ascorbate is a key antioxidant because it neutralizes free radical species. Ascorbate has a low reactivity with common cellular oxidants including hydrogen peroxide, and it is most likely to react with hydrogen peroxide breakdown products.

Superoxide, singlet oxygen, hydroxyl radical, hypochlorous acid, and the iodinating activity of the MPO/H_2O_2/iodide system are all neutralized by ascorbic acid, although it does not scavenge or neutralize H_2O_2 per se (Halliwell, 1999).

2.3.7 Vitamin E regeneration by vitamin C

Vitamin C was found to improve the antioxidant potency of vitamin E in lard and cottonseed oil as early as 1941, and in 1968, Tappel proposed that vitamin C could regenerate vitamin E from the vitamin E radical, which is produced when vitamin E quenches a lipid peroxyl radical.

2.3.8 Pro-oxidant effect of vitamin C

In vitro, ascorbic acid is considered to act as a pro-oxidant. For decades, ascorbic acid and copper or iron mixtures have been used to cause oxidative modifications in lipids, proteins, and DNA (Halliwell and Gutteridge, 1999). By reducing ferric Fe3+ to ferrous Fe2+ ions (and Cu2+ to Cu+), ascorbic acid may reduce hydrogen peroxide (H_2O_2) to hydroxyl radicals, which can lead to oxidative damage. However, due to efficient iron sequestration by metal binding proteins like ferritin and transferrin, these vitamin C-mediated Fenton reactions should be regulated in the human body. As a result, it's been suggested that the prooxidant effect isn't important in vivo (Halliwell and Gutteridge, 1999). Nonetheless, vitamin C supplements are not recommended in

people with elevated iron levels or in pathological conditions like haemochromatosis or thalassemia, which are also associated with iron overload (Herbert *et al*., 1996). Indeed, without the use of free transition metal ions, vitamin C has been shown to cause the decomposition of lipid hydroperoxydes to genotoxic bifunctional electrophiles in vitro. In the presence of free Fe3+ or Cu2+, in vitro L-ascorbic acid will promote the production of reactive oxygen species (OH, O^2-, H_2O_2, and ferryl ion). The ability of L-ascorbic acid to reduce Fe3+ or Cu2+ to Fe2+ or Cu+, respectively, and to reduce O2 to O2- and H_2O_2 results in this pro-oxidant action. However, no pro-oxidant activity contributed to L-ascorbic acid was evident after summarizing the findings of in vivo studies that assessed the oxidation of LDL, lipids, and proteins (Halliwell and Gutteridge, 1999).

2.3.10 Vitamin C in Human Diseases:

Low levels of plasma vitamin C have been linked to oxidative stress in a variety of conditions, including cataracts, diabetes, cancer, HIV infection, smoking habits and SLE (systemic lupus erythematosus). Vitamin C's potential role in cancer treatment and prevention has piqued researchers' interest. Thus, scientist can not categorically say that vitamin C supplements is useful in cancer prevention if they can prevent the development and/or facilitate the repair of pre-mutagenic oxidative DNA lesions. Furthermore, an early study found that daily supplementation with high doses (grams) of vitamin C improved the survival time of terminal cancer patients, suggesting that vitamin C may have significant anticancer properties. Vitamin C destroys or delays the growth of many tumor cell lines, as well as increasing the cytotoxicity of radiosensitizing drugs. Several studies have also shown that cancer cell lines are more susceptible to vitamin C than non-malignant cell lines. In terms of cancer prevention, numerous epidemiological studies have linked a diet high in fruits and vegetables (basically antioxidants) to a lower risk of a variety of cancers (Levine *et al*., 2001).

Consumption of fruits and vegetables has also been linked to a lower risk of cardiovascular disease (CVD). Low levels of vitamin C in the blood have been linked to CVD death, and it has been suggested in the literature that vitamin C can protect against CVD through a variety of mechanisms. Endothelial dysfunction is linked to atherosclerosis, hypercholesterolemia, hypertension, diabetes, and smoking. Vitamin C increases endothelium-dependent vasodilation, which helps to avoid atherosclerosis, hypercholesterolemia, hypertension, diabetes, and smoking. This seems to be due to vitamin C's ability to increase atheroprotective nitric oxide levels (NO). Vitamin C has been shown to increase endothelial NO synthase activity by maintaining its cofactor, tetrahydrobiopterin, in a reduced state and thereby increasing its intracellular availability (Levine *et al*., 2001).

2.3.10 Dietary requirement of ascorbate

There has been a lot of controversy about the safety of high doses of ascorbic acid and the amount of vitamin C that should be ingested for optimal health. Amounts ranging from 30 to 10,000 mg per day have been prescribed by various authorities. A daily dose of 10 mg of ascorbic acid is normally sufficient to relieve and treat clinical symptoms of scurvy in humans, although it does not always provide an adequate supply of the vitamin. For adult nonsmokers, the RDA for vitamin C is 60 mg. It is well known that smokers have lower vitamin C levels in their blood. As a result, the RDA for vitamin C for smokers was set at 100 mg in 1989. Vitamin C supplementation is relatively healthy from a toxicological standpoint, except at megadose levels (i.e., 1-4 g/d) (Levine *et al*., 2001). Vitamin C is currently recommended in dietary daily allowances of 90 mg for men and 75 mg for women. Ascorbate starts to appear in the urine at vitamin intakes of around 60 mg/d in both genders. Daily intakes of 250 mg or more are needed to saturate ascorbate concentrations in plasma and white blood cell contents (Levine *et al*., 2001).

2.3.11 Role of Ascorbate in Male Reproduction

Testicular differentiation, integrity, and steroidogenic functions all require ascorbic acid (Salem *et al*., 2001). Furthermore, ascorbic acid protects sperm from oxidative damage by acting as an antioxidant in the sperm (Yousef, 2005). Because of its importance, ascorbic acid levels in the testes remain stable (Mukkadum, 1980).

Ascorbic acid plays a key role in the fertility of men and animals as it safeguards spermatogenesis, retains the viability of sperms, prevents sperm agglutination, and raises the testosterone in serum (Fernandes *et al*., 2011). Ascorbic acid supplementation has also been shown to increase total sperm output and sperm concentration (Kini *et al*., 2009). Ascorbic acid seems to positively affect testicular and erectile functions in unhealthy and healthy persons with reproductive complications (Sonmez & Demirci, 2003). Some evidence suggests that testicular and erectile dysfunction is linked with low Ascorbic acid levels in plasma (Fazeli *et al*., 2010). In context, in infertile men with asthenozoospermia, seminal fluid had lower Ascorbic acid levels and greater ROS values than those of fertile ones (Lewis *et al*., 2013).

Vitamin C is a strong antioxidant in the testes (Augustine *et al*., 2005). It inhibits sperm agglutination by neutralizing reactive oxygen species (ROS) (Dawson *et al*., 1992). It acts as an electron donor for redox systems by protecting DNA from hydrogen peroxide-induced damage, recycles vitamin E and prevents lipid peroxidation (Jedlinska-Krakowska *et al*., 2006). Taking 200 mg of vitamin C orally for two months, along with vitamin E and glutathione, substantially decreased hydroxyguanine (8-OH-dG) levels in spermatozoa while also increasing sperm count (Kodama *et al*., 1997). TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) positive spermatozoa were decreased after two months of daily oral vitamin C and E supplementation (Greco *et al*., 2005a). Furthermore, antioxidant therapy has been related to a substantial increase in clinical pregnancy and implantation rates. During density gradient

separation, vitamin C and E were added to the sperm preparation media to protect sperm from DNA damage (Hughes *et al*., 1998; Greco *et al*., 2005b; Agarwal *et al*., 2008). Vitamin C levels were found to be lower in men who had a lower sperm count, motility, or an increased irregular sperm count. The concentration of epididymal sperm and serum testosterone in rats increased when the vitamin C concentration was high. Supplementing with vitamin C tends to increase the consistency of sperm (Sönmez *et al*., 2005). Gentamycin causes oxidative stress via free radical formation, lipid peroxidation, and decreasing antioxidant levels. Vitamin C supplementation reversed these results, meaning that this vitamin plays a direct role in oxidative damage defense (Narayana, 2008). Furthermore, some toxins, such as cisplatin, which lower vitamin C levels, cause an increase in reactive oxygen species (ROS) and damage germ cells, resulting in a significant drop in sperm count (Narayana, 2008) and a higher incidence of DNA damage (Thiele *et al*., 1995). Vitamin C significantly, but not completely, protects against cisplatin-induced testicular damage (Narayana *et al*., 2009). Levels of 8-oxo-7,8-dihydro-2´-deoxyguanosine, a major product of oxidative damage, were measured in DNA isolated from human sperm. According to the findings by Fraga *et al*., 1991, high levels of this substance were linked to low seminal plasma vitamin C levels. Dietary vitamin C has been known to play a crucial role in protecting male germ cells from oxidative damage since the 1920s. In guinea pigs, a low-vitamin C diet induces massive degeneration of the seminiferous epithelium, with desquamation into the testis tubule lumen (Lindsay & Medes, 1926). As compared to plasma, seminal fluid has a higher concentration of vitamin C (400 vs. 60 mM), but infertile seminal plasma has a lower concentration (Lewis *et al*., 1997). After exposure to toxic molecules like chrome and cadmium, the amount of vitamin C in the testes decreases drastically. While ascorbic acid levels in the testis are unaffected during development, an ascorbic acid deficiency in the testis of chrome-exposed animals disrupted spermatogenenesis, resulting in a dramatic drop in

sperm count (Augustine *et al*., 2005) and significantly increased rates of sperm abnormality. Vitamin C and vitamin E supplements, on the other hand, decreased lipid peroxidation and increased sperm count in cadmium-induced mice (Achayra *et al*., 2008). Germ cell detachment has been shown to occur in mixed cultures of Sertoli and germ cells in response to some known in vivo testicular toxins. Such cultures were also treated with the oxygen radical generating system of xanthine/xanthine oxidase (Anderson & Francis, 1993). This treatment caused an increase in germ cell detachment, which was reduced by vitamin C but not by vitamin E at the doses used. These findings suggest that supplementing with vitamin C may protect reproductive somatic cells from toxins that act through oxygen radical mechanisms (Anderson & Francis, 1993). Other research into the relationship between vitamin C and germ cell development has found that serum and vitamins A, C, and E improved germ cell differentiation in in-vivo testis cultured from infant rats (Steinberger & Steinberger, 1966). Vitamin C was shown to be necessary for maintaining the physiological integrity of the androgen target reproductive organs by Chinoy *et al*. (1986). Since vitamin C is needed for the hydroxylation of collagen, abnormal spermatogenesis and infertility are linked to a malfunction of the testicular cell-cell interaction (Yazama *et al*., 2006). Vitamin C deficiency in guinea pigs resulted in a decline in the weight of reproductive organs, according to Sapra *et al*. (1987). Other research found that certain toxic compounds reduced the weight of the testis and accessory sex organs, and that supplementing with vitamin C reversed this reduction (Das *et al*., 2002).

2.4 Zinc

Zinc (Zn) is a trace element that can be found almost everywhere. It is one of the most essential trace elements in the body, and it is needed for microorganisms, plants, and animals to grow and develop. It is present in relatively high concentrations in all body tissues and secretions, with 85 percent of total body zinc found in muscle and bones, 11 percent in the skin and liver, and the

remainder in all other tissues, with the highest concentrations in the prostate and areas of the eye. The average adult body contains around 1.4–2.3 g of zinc (Calesnick and Dinan1988; Stefanidou *et al*. 2006; Prasad 2009; Bhowmik *et al*. 2010). After iron, zinc is the second most abundant transition metal ion in living organisms. Moreover, if hemoglobin-bound iron is not taken into account, Zn becomes the most abundant transition metal ion the body (Vasak and Hasler 2000). Zinc is the only metal that serves as a cofactor for over 300 enzymes (Rink and Gabriel 2000), and it plays a key role in the structural stability of a large number of proteins, including signaling enzymes at all stages of cellar signal transduction and transcription factors (Beyersmann, 2002). Zinc is also needed for zinc finger protein structural stability (Zfp). Zinc-containing proteins, also known as zinc cluster proteins, are transcription factors classified as Class I Cys2His2 (C2H2) proteins, Class II Cys4 (C4) zinc finger proteins, and Class III (C6) zinc finger proteins. Zinc is the key component of Zfps, which are the largest and most diverse superfamily of nucleic acid-binding proteins. They interact with zinc-binding domains such as zinc fingers, RING fingers, and LIM domains to play important roles in transcriptional control of the cellular metabolic network (Joazeiro and Weissman 2000; Kadrmas *et al*., 2004; Chasapis and Spyroulias, 2009; Zhao and Bai 2011). Zinc, unlike other transition metal ions like copper and iron, does not undergo redox reactions because its D-shell is filled. It is a biologically significant trace element that is necessary for cell growth, differentiation, homeostasis, connective tissue growth and maintenance, DNA synthesis, RNA transcription, cell division, and activation. Wound healing, taste acuity, immune system function, prostaglandin production, bone mineralization, proper thyroid function, blood clotting, cognitive functions, fetal growth, and sperm production are all dependent on it. Zinc preserves normal prostate function and has a significant role in testosterone secretion. It controls body fluid pH, encourages the production of

collagen to make hair, skin, and nails, and it aids memory and mental growth (Bhowmik *et al*., 2010).

The seminal fluid contains high volume of zinc, which plays a number of roles in the sperm's functional properties. It has been proposed that Zn acts as an anti-inflammatory factor and is involved in the oxidative metabolism of sperm. Zn plays a variety of important roles in spermatozoa physiology, including lipid versatility and sperm membrane stability (Chia *et al.*, 2000). It's also essential for conception and embryonic implantation because it controls capacitation and the acrosome reaction of sperm (Eggert-Kruse *et al*., 2002).

The Zn finger motif Cys2/His2 of P2 protamine plays an important role in the prevention of transcription by sperm chromatin stabilization and in the inhibition of oxidative damage in human spermatozoa and possibly in the rest of mammalian organisms. In mammalian spermatozoa, proteins containing zinc and selenium are involved in regulating the amount of reactive oxygen species (ROS). Contreras *et al*. reported that Zn supplementation concentration of 200 ppm has a negative effect on boar sperm DNA content, which they conclude is linked to spermatozoa's ability to accumulate Zn during spermatogenesis. They also observed a positive association between the percentage of sperm DNA fragmentation and the Zn concentration in spermatozoa (Garcia-Contreras *et al*., 2011; Plum *et al.*, 2010; Sauer *et al*., 2016, Wani *et al*., 2017). Weak Zn nutrition was considered to be a powerful risk factor for low sperm quality and idiopathic male infertility in a previous study by Colager *et al*., 2009.

2.4.1 Roles of Zn in health

The human body contains 2-3 grams of zinc, with approximately 90% of it concentrated in muscles and bones. Prostate, liver, and gastrointestinal tract are among the nearby organs; kidney, skin, lung, adrenals, brain, heart, eyes, and pancreas also contain substantial quantities of Zn. Zn is critical for men's health for a variety of reasons. Typical examples include assisting

immune function, encouraging healthy cell development, and playing a role in prostate health, sexual health, and testosterone hormone levels. Zn has been shown to play an important role in reproductive function (Plum *et al.*, 2010; Wong *et al*., 2001). Hosseinzadeh Colager *et al*., found that Zn levels in fertile groups were substantially higher than in infertile groups' seminal plasma. Fertile men had a Zn level of 14.08 2.01, while infertile men had a level of 10.322.98 (mg/100 ml) (Colager *et al.*, 2009).

There are many reports for Zn-physiological roles in human, including its role in gonads and some enzyme- functions, treatments of some diseases, and better function of apoptosis and immune systems. Some of these Zn-physiological roles are indicated below.

2.4.2 Zinc's Functions in the Male Reproductive System

Zinc concentration in the testes: Zn levels in vertebrates' testicles are comparable to those in the liver and kidney. Furthermore, there are some studies that show Zn can minimize testis injury caused by heavy metals, fluoride, and heat (Boran and Ozkan, 2004). Zn is accumulated in the testis during early spermatogenesis and may play an important role in spermatogonial reproduction and germ cell meiosis (Elgazar *et al.*, 2005). During spermatogenesis, Zn assembles primarily in germ cells, and its concentration in the testis increases. As a result, neither interstitial tissue nor sterol cells contain Zn.

This is also the reason why Zn deficiency impedes spermatogenesis which is a major reason for sperm abnormalities (Yamaguchi *et al.*, 2009). Zinc is necessary for making the outer membrane and tail of the sperm and also important for sperm maturation. Zinc supplements have been shown to improve sperm count, motility, form, function, quality and fertilizing capacity (Deborah and Haim, 2020).

Zinc and male steroid hormones: Literatures from different researchers have revealed that zinc is necessary for normal functioning of the hypothalamus-pituitary-gonadal axis in men. Since Zn

plays such an important role in male reproductive capacity, it's critical to understand which andrological variations are most vulnerable to Zn depletion. Care would be accelerated if those factors were evaluated in clinical cases of suspected Zn deficiency. Zn impacts male fertility in a number of ways. Low zinc levels have a detrimental impact on testosterone levels in the blood (Hunt *et al*., 1992).

Thyroid hormones play a variety of roles in the body, including metabolism, growth, and body temperature regulation. Proper thyroid function is being mediated by zinc by causing thyroid-releasing hormones to be generated in the brain. Men who are deficient in zinc may be unable to produce enough of these hormones.

This may also have an effect on testosterone levels (Yan *et al.*, 2010). Adult males who denied Zn displayed a disorder of testosterone synthesis in the Leydig cell, according to a clinical review, because Zn plays a key role in the 5α reductase enzyme, which is required for the transformation of testosterone into a biologically active form, 5α dihydro testosterone (Ali *et al.*, 2007). In the same way as Zn affects a man's fertility, it also affects his sex drive and long-term sexual health. The effect of Zn on testosterone hormone levels was investigated by Yan *et al*., 2010, and it was discovered that catching Zn supplements improved testosterone production (Yan *et al.*, 2010). Seminal Zn is assumed to be a prostatic function predictor, but the function of Zn in seminal plasma and sperm is uncertain. The majority of Zn secreted from the prostate in humans appears to target seminal vesicle proteins. According to other studies, the Zn and albumin secreted by the prostate form a complex that protects the cells by covering the sperm (Hunt *et al.*, 1992). The Leydig cells, late type B spermatogonia, and spermatids are the key sources of Zn in the testes.

It's also essential for the Leydig cells' testosterone production and secretion. Zn appears to play a significant role in the physiology of spermatozoa in human sperm. On the other hand, there is

compelling evidence that zinc deficiency causes primary testicular failure, reduces luteinizing hormone receptor activity, reduces steroid synthesis, and causes oxidative stress-induced Leydig cell damage (Croxford *et al*., 2011).

Prostate gland and zinc: The prostate has a high Zn concentration as compared to other tissues and body fluids. In reality, Zn is a prostatic function marker. Other roles include controlling spermatozoa functions, serving as a cofactor for most enzymatic reactions, and assisting in the preservation of sperm motility (Wong *et al.*, 2001). Zn is discharged from the prostate as a downward molecular weight complex with citrate or attached to glycoproteins of the sperm vesicles, demonstrating that biologic Zn therapy improves sperm motility and that biologic Zn supplementation was an effective tool for treating infertile males with chronic prostatitis (Deng *et al*., 2005).

Roles of Zn in quality and function of sperm: Evidence suggests that seminal Zn has an important role in the physiologic functions of the sperm and thatitreduced levels result in low seminal quality and subsequent chances of fertilization (Colager *et al*., 2009). Zn is needed for sexual maturity and the onset of estrus. Zn is needed for preserving the lining of the reproductive organs, as it plays a role in epithelial integrity (Colager *et al*., 2009). It also aids in the stabilization of the cell membrane and nuclear chromatin of spermatozoa in seminal plasma. It can play a role in the progression of capacitation and acrosome reaction by acting as a regulator. Human seminal plasma has a higher Zn concentration than other tissues (Cheah *et al*., 2011). Zn is needed for spermatogenesis, according to research. It has an important role in the production of the testis and the physiologic functions of sperm (Colager *et al*., 2009). Zn plays a number of roles during the spermatogenesis process. Zn is important in the initiation of spermatogenesis, for example, since it participates in ribonuclease activities, which are highly active during spermatogonia mitosis and spermatocyte meiosis. Spermatids develop a tail and motility during

spermatogenesis, thanks to a formed mid-piece that connects the head compartment to the tail. Seminiferous tubules go through this physical maturation process. The epididymis is where sperm mature and are deposited. Zn is highly concentrated in the tail of mature spermatozoa at the end of spermatogenesis and is involved in sperm motility (Cheah *et al.*, 2011).

Dynamism of Zn during sperm expansion are far from simple, its significance in normal spermatogenesis and sperm suitability is well understood. Foresta *et al*. elucidated the expression of Zn transferor at the testicular, epididymis, and ejaculate levels, as well as the function of Zn content in sperm communication at various stages of sperm existence (Foresta *et al*., 2014). Optimal Zn concentrations in seminal plasma have also been linked to increased sperm concentration in the ejaculate (Chia *et al*., 2000), high motility, viability (Roy *et al*., 2013), and increased antioxidant activity (Roy *et al*., 2013). (Oliva *et al*., 2009). Low Zn levels have been shown to disrupt the testes fatty acid composition and thus, affect normal testis endocrine regulation (Merrells *et al*., 2009). Zn is linked to lipid catabolism in the sperm middle piece, which provides energy for spermatozoa motion. Zn influences spermatozoa oxygen consumption in seminal plasma, as well as head-tail attachment and detachment, and nuclear chromatin condensation and decondensation. Zn is required for normal spermatogenesis and sperm physiology; it protects sperm genomic integrity and secures sperm head-to-tail attachment (Roy *et al*., 2013).

2.4.3 Mechanisms of Zn transport in cells and at the molecular level

Zn homeostasis and its biological importance have been the topic of numerous studies. Chemistry as a field has looked into recent advances in cell biology and the existence and function of free or unstable Zn in cellular responses, specifically its neurotransmitter activity in synaptic vesicles (Sensi *et al.*, 2009). On a cellular basis, Zn is found in the nucleus in 30–40%

of cases, the cytosol in 50% of cases, and membranes in the remaining cases (Kambe *et al.*, 2015).

Zn and seminal plasma proteins interact: Zn ions are bound to seminal plasma proteins in mammals and protect sperm chromatin stability. This ion is involved in the formation of S-Zn-S type bonds in protamine structure, which helps to keep chromatin stable (Schneider *et al.*, 2009). The human prostate gland secretes zinc in two forms: free and in complexes with high molecular weight proteins (Mogielnicka-Brzozowska and Kordan, 2011). Zn is abundant in the tails of mature spermatozoa, where it is linked to sulfhydryl groups and disulfide linkages (Schneider *et al*., 2009). By forming a specific number of SH-Zn-SH structures in the sperm nucleus, Zn controls disulfide cross-links (Khosronezhad *et al*., 2015). Semenogelins are the most well-known Zn-binding proteins found in human seminal plasma. They are involved in the formation of coagulum, DNA stability control, sperm movement inhibition, and antibacterial action. These proteins also resist capacitation by hyperpolarizing sperm plasma membranes (Mogielnicka and Kodan, 2011).

The method of transporting Zn is as follows: Despite the importance of zinc for spermatogenesis, testicular function, and fertility, little is known about how the testis obtains Zn from circulation, how Zn is transferred into maturing gametes through the testis during spermatogenesis, or how marginal Zn deficiency affects this process. For cellular Zn transport, there are three main mechanisms.

One of them is the transport of proteins from the Zip-family across the plasma membrane, as well as the export of proteins from the ZnT-family. The third mechanism is accomplished by transporter-mediated decomposition into intracellular organelles such as endoplasmic reticulum, Golgi, and lysosomes, and the fourth mechanism is accomplished by Zn-binding proteins such as metallothionein (Plum *et al.*, 2010).

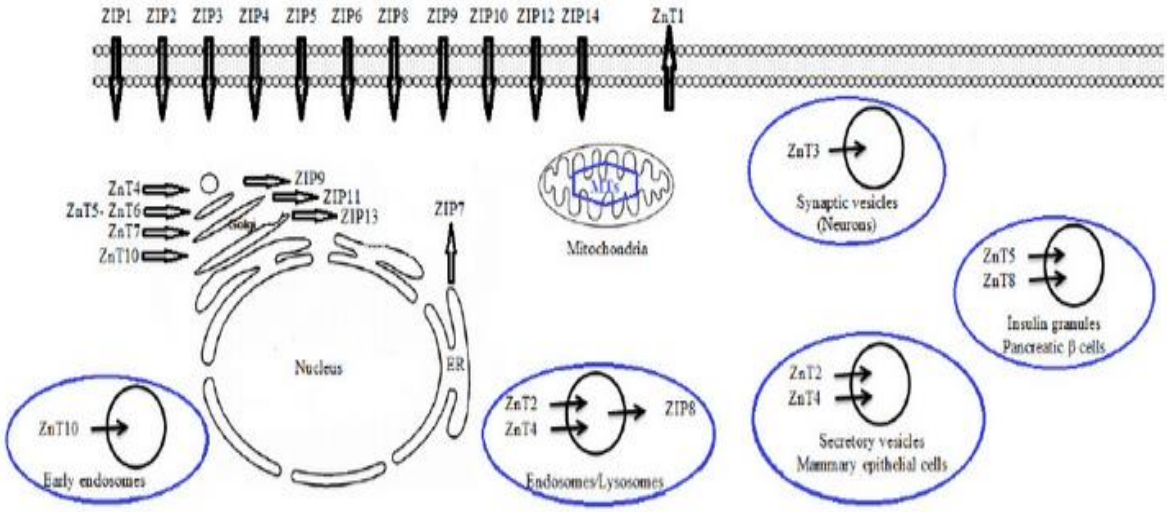


Figure 2.6: Scheme for zinc dissemination in cells: This scheme isa mixed one obtained from previous studies (Haase *et al.*, 2009, Kambe *et al*., 2015, Tamano *et al*., 2016)

Schematic illustration above (figure 2.6) shows the motion of Zn through physiological membranes by two groups of Zn transfer proteins. These proteins function together to keep the right amount of Zn in the cells. The SLC30 (ZnT) and SLC39 protein families are the ones in question (Zip). The ZnT-transferor increases the volume of Zn in cellular effluent or secretion into intracellular organelles. Zip transporters, on the other hand, promote extracellular or intra-organ Zn entry into the cytoplasm (Elde, 2006). 10ZnTand14 Zip transporters have previously been established, but only a few studies have tested human primary tissues for Zn transporter expression patterns (Foresta *et al*., 2014). Zip proteins make it easier to carry Zn into the cytoplasm, whether from the extracellular system or from an intracellular membrane.

2.5 Chlorpromazine

Chlorpromazine has been used overtime for the treatment of both acute and chronic psychoses including schizophrenia and manic-depressive illness. Chlorpromazine (CPZ), marketed under the trade names Thorazine and Largactil among others, is an antipsychotic medication. It is primarily used to treat psychotic disorders such as schizophrenia (Healy, 2004). Other uses include the treatment of bipolar disorder, nausea and vomiting, anxiety before surgery and hiccups that do not improve following other measures (John, 2019). Chlorpromazine antipsychotic activity is through blockage of dopamine (D2) receptors in the mesolimbic pathway of the brain, over activity of which is understood to be responsible for the symptoms of schizophrenia (Healy, 2004). Anti-dopaminergic extrapyramidal syndromes (peirre, 2005), dry mouth, blurred vision, and urinary retention (anticholinergic) (pilliai *et al.*, 2006), neuroleptic dysphoria, blood pressure disturbances, temperature, and muscle regulation (neuroleptic malignant syndrome), reduced libido, erectile impotence, and ejaculation inhibition in male patients have also been related the effects of chlorpromazine on the body (Greenberg, 1971). Chlorpromazine has been reported to induce changes in human erythrocytes (Freeman and Spirtes, 1963). Bilde *et al.,* 1977, studied the effects of Chlorpromazine pretreatment on kidney viability and function after warm ischemic damage and reported that Chlorpromazine has protective effects on kidney. El-Ashmawy and Youssef, 1999 also reported its antagonistic/protective effects on testes damaged with cadmium. Lewis and Evans, 1969 observed that Chlorpromazine has depressant effect on Rapid eye movement (REM) sleep.

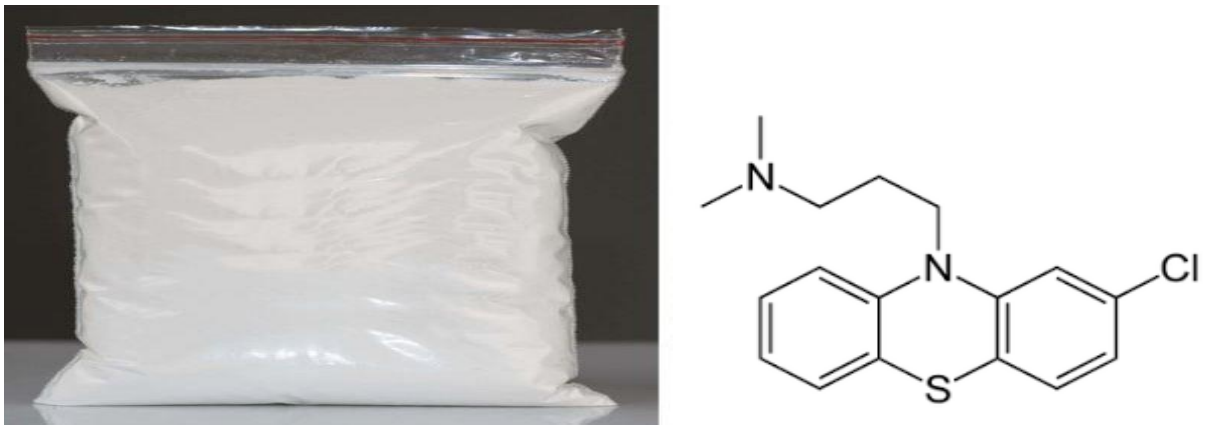


Figure 2.7 Shown image and chemical structure of Chlorpromazine (Anz *et al*., 2011).

2.5.1 Systemic Effects of Chlorpromazine

DeLorey *et al.*, 2008 reported the comparative effect of *Rauwolfia vomitoria*, chlorpromazine and reserpine on social behaviour, nesting behaviour, a reflection of the social behaviour in mice, Chlorpromazine dose-dependently decreased the nesting score. Thus, higher doses of chlorpromazine caused greater degree of social loss when compared to lower doses. These results are consistent with the work of (Li *et al*., 2005) who reported that Chlorpromazine depressed nesting behaviour in mice.

Significant reductions in spermatogenesis indexes were observed following chlorpromazine administration in both animal models and humans, but significant improvements in sperm quality were observed following zinc administration, particularly in cases of established infertility. Zinc intake improves sperm count, mobility, and fertilizing ability while lowering DNA damage, structural abnormalities in sperm, and antibodies to sperm, all of which can degrade sperm quality (Omu *et al*., 2008).

Male and female Sprague-Dawley strain rats were used to test the effect of chlorpromazine (CPZ) administration prior to insemination on reproduction over three generations. Male and female rats were given CPZ doses orally every day for 9 weeks before mating, with the following results: In F0 females, the estrous period lasted longer. The number of living fetuses

and newborns, as well as the incidence of insemination and pregnancy in F0 males and females, all showed a downward trend. The body weight of the rats in the F0 and F1 generations decreased as the dose of CPZ increased, but the F2 generation's body weight increased. The administration of 100 mg/kg CPZ to rats in the F0 and F1 generations affected the wet weight of their main organs. The liver, kidney, adrenal, pituitary, testis, and prostate glands weight in both male and female rats were increased, while the weights of the pituitary and uterus were decreased in females. These findings indicate that giving CPZ to the parent rats before mating has an impact on the F0 and F1 generations' reproduction (Keiya, 1990).

2.5.2 Chlorpromazine's Mechanism of Action

Chlorpromazine is a powerful antagonist of D2 dopamine receptors and other dopamine receptors including D3 and D5. It also has a high affinity for D1 receptors, unlike most other drugs in this class. When these receptors are blocked, neurotransmitter binding in the forebrain is reduced, resulting in a variety of results. Since dopamine is unable to bind to a receptor, it creates a feedback loop through which dopaminergic neurons release more dopamine. As a result, patients will find an improvement in dopaminergic neural activity when they first start taking the drug. The neurons' dopamine output will gradually decrease, and dopamine will be removed from the synaptic cleft. Neural activity plummets at this stage, and the continued blockade of receptors exacerbates the problem. Chlorpromazine acts as a blocker (antagonist) on a variety of postsynaptic and presynaptic receptors.

Dopamine receptors (subtypes D1, D2, D3, and D4) in the mesolimbic dopamine system account for various antipsychotic properties on productive and unproductive symptoms, while blockade in the nigrostriatal system causes extrapyramidal impact. Serotonin receptors (5-HT1 and 5-HT2) have anxiolytic and anti-aggressive properties, as well as a reduction in extrapyramidal side effects, but they also cause weight gain and ejaculation problems. α 1 and α2-adrenergic

receptors are responsible for sympatholytic properties, reflex tachycardia, vertigo, sedation, lowering of blood pressure; hyper salivation and incontinence as well as sexual dysfunction while histamine 1 receptors responsible for sedation, antiemetic effect, vertigo, and weight gain Also associated with weight gain as a result of blockage of the adrenergic alpha 1 receptor) M1 and M2 muscarinic acetylcholine receptors (causing anticholinergic symptoms such as dry mouth, blurred vision, constipation, difficulty or inability to urinate, sinus tachycardia, electrocardiographic changes and loss of memory, but the anticholinergic action may attenuate extrapyramidal side effects). The presumed effectiveness of the antipsychotic drugs relied on their ability to block dopamine receptors. This assumption arose from the dopamine hypothesis that maintains that both schizophrenia and bipolar disorder are a result of excessive dopamine activity. Furthermore, psychomotor stimulants like cocaine that increase dopamine levels can cause psychotic symptoms if taken in excess (Suzanne *et al*., 2014).

Chlorpromazine and other typical antipsychotics are primarily blockers of D2 receptors. In fact an almost perfect correlation exists between the therapeutic dose of a typical antipsychotic and the drug's affinity for the D2 receptor. Therefore, a larger dose is required if the drug's affinity for the D2 receptor is relatively weak. A correlation exists between average clinical potency and affinity of the antipsychotics for dopamine receptors (Girault *et al*., 2004). Chlorpromazine tends to have greater effect at serotonin receptors than at D2receptors, which is notably the opposite effect of the other typical antipsychotics. Therefore, chlorpromazine with respect to its effects on dopamine and serotonin receptors is more similar to the atypical antipsychotics than to the typical antipsychotics (Girault *et al*., 2004).

2.5.3 Role of Chlorpromazine in male reproduction

Antipsychotic medications have been studied for their effect on prolactine (PRL) levels in rats and rabbits. In rats given a single subcutaneous injection at low concentration, PRL levels increased more than fourfold within 1 hour and remains elevated for 6 hour with chlorpromazine (Drobnis & Nangia, 2017). In one interesting study of loxapine and its isomer isoloxapine, male rats given a single injection of approximately 0.001–0.1× human equivalent dose (HED) of loxapine displayed high D2 receptor occupancy and profoundly increased PRL at doses of 0.01× HED or higher; while isoloxapine, did not have a detectable effect on PRL levels (Drobnis & Nangia, 2017). PRL levels increased for approximately 2.5 HED [oral] haloperidol, 0.3 HED [oral] pimozide, 0.1 HED [oral] sulpiride, and HED [oral] chlorpromazine when administered intravenously to awake, freely moving male rabbits (Drobnis & Nangia, 2017). In rats given antipsychotic medications, the time between intromission and ejaculation is also lengthened. Approximately 0.2 HED [oral] of thioridazine induced increased ejaculatory latency in a rat study conducted by Drobnis & Nangia, 2017.

Antipsychotics have been shown to have detrimental effects on spermatogenesis and fertility in rats in experiments at HED. In one study, rats given intraperitoneal injections of olanzapine for 45 days at doses of 1, 2, 4, or 8 HED [oral] had lower testosterone levels, lower testes, epididymis, and prostate weights, and histological degeneration of the seminiferous tubule epithelium at the higher doses (Drobnis & Nangia, 2017). In rats, pharmacological doses of antipsychotic drugs were tested for fertility effects. Haloperidol administered orally to rats at doses ranging from 1.6– 32 HED [oral] for up to 9 weeks had no effect on mating activity, and only the highly pharmacological doses produced adverse effects such as decreased testis weight, histological disruption of spermatogenesis, and decreased fertility (Drobnis & Nangia, 2017). In a study of male rats treated with approximately 3–10 HED [oral] of fluphenazine by

subcutaneous injection for 2 months, PRL was higher and gonadotropin levels were lower at all doses relative to vehicle-treated controls. Lower fertility and DNA fragmentation were observed at higher doses, but caudal epididymal sperm characteristics were unaffected. Antipsychotic medications have been tested in vitro on the contractility of rat reproductive tissue, with conflicting findings (Drobnis & Nangia, 2017).

Williams *et al.,* 2004 reported a significant increase in serum prolactin and significant decrease in testosterone and Luteininzing Hormone after 10 mg/kg.bw Chlorpromazine treatment in rat. Ali *et al.,* 2011 observed that chlorpromazine result in loss of follicle by atresia and delay the onset of puberty in immature female rat. Raji *et al.,* 2005 reported that chlorpromazine at higher concentration causes severe abnormalities in the seminiferous tubule.

2.6 Plant of study (*Rauwolfia vomitoria)*

Apart from using synthetic antipsychotic drugs like CPZ and reserpine, traditional herbs like *Rauwolfia vomitoria* has being reported to be a potent psychotic drug.

Traditional ways of treating ailments has moved man closer to natural product being easy to assess, cheap and affordable and very effective. One of the commonly used plant in Africa (Nigeria to be precise) in the treatment of psychosis is *Rauwolfia vomitoria. Rauwolfia vomitoria* is widely used in traditional medicine in Africa and has become a major source a number of compounds used in the pharmaceutical industry, particular reserpine, and deserpidine etc. *Rauwolfia vomitoria* is a shrub or a tree that varies widely in height; it usually grows from 0.5-20 meters tall, though specimens up to 40 meters have been recorded. The tree is harvested from the wild for local use as a medicine and source of materials. It is commonly harvested from the wild and exported to places such as Europe as a source of compounds, especially reserpine, for the pharmaceutical industry. It is found in areas where the mean annual temperature is around 26°C and the mean rainfall around 1,375mm. Dry season samples of the roots have been found to

contain a higher content of alkaloids than wet season samples. *Rauwolfia vomitoria* has flowers of two different shoes: slender flowers and robust flowers. The slender flowers occur on a diploid plants, while the robust flowers occur on hexaploid plants. The root bark of diploid plants contains less reserpine than that of hexaploid plants. The *Rauwoulfia vomitoria* contains different indole alkaloids, most of which issue in very small amounts and several are disputed. The leaves contains 03-0.8% total alkaloids; the stem bark about 0.6%;the roots 0.15-0.2%;and the root bark From 1.5-2%. The alkaloids can be grouped into 5 main groups: Yohimbine and derivatives (including reserpine and deserpidine), heteroyohimbine type (including ajmalicine, reserpine and deserpidine), Sarpagane derivatives (including surpagine), dihydro-indole type (including ajmalinr), anhydronium bases (including alstonine, serpentine and serpenticine). Reserpine is a well-known antihypertensive, antipsychotic and sedative (Michelle and Mayur, 2020). All these alkaloids have being modified into chemical compounds called phytochemicals of *Rauwoulfian vomitoria.* Other phytochemicals in *Rauwolfia vomitoria* includes Zn, Ca, Fe, Mg, Mn, As, Cu, Vit C etc. (Jaya and Amit, 2016).

2.6.1 Study of *Rauwolfia vomitoria* on body organs

Mokutima *et al*., 2009 who observed significant increase in body weight loss in animal treated with *R.vomitoria* at high doses (500 and 600 mg/kg). This indicates that administration of *R.vomitoria* at defined doses could affect fat and carbohydrates metabolism. Reduction in the body weight might be due to the presence of alkaloids in the extract. Several lines indicated that alkaloids could alter cells growth (Hiroshi *et al*., 2004), and lead to inhibit food intake. Therefore the amount of nutrients absorbed by animals treated with aqueous extract of *R.vomitoria* would not have been sufficient to properly cover their need for growth.

The obtained data in the work also showed a significant weight increase of testis in high doses, seminal vesicles and prostate in all doses. Increase in the weight of testis and accessory sex

organs directly supports the increase availability of androgen (Naseem *et al*., 1998) since testis, seminal vesicle and prostate where significantly related respectively to testicular cholesterol and testosterone level at the same doses. It is well known that androgen plays an important role in the maintenance of accessory sex organs and spermatogenesis (Guido *et al*., 2010) Studies have shown that administration of exogenous testosterone enhanced growth of the androgen-dependent organs (Dewan *et al*., 2002) Other works revealed that the total testicular amount of immune detectable androgen receptors protein in long term testosterone deprived rats was very low when compared to that in control rats or rats with testosterone-implants (Blok *et al*., 1992) Any change in the circulating androgen would affect structural and functional integrity of reproductive tissues (Chinoy *et al*., 1986) and thereby lead to alteration in sperm motility and metabolism. The work shows the significant increase of testosterone level in blood indicates the increase of androgen level in all *Rauwolfia vomitoria* doses treated animals. Because of an increase androgen concentration in animals treated with Rauwolfia vomitoria extract, the ability of the extract fed rats was sufficient for the maintenance of maturation of sperm cells of the epididymis (Dieudonne *et al*., 2014) and the weight gain of gonads and accessory organs. It is important to note that androgen deficiency not only suppresses spermatogenesis, resulting in low sperm concentration, but also alters the epididymal milieu, making it hostile to spermatozoa physiological maturation and survival (Rao *et al*.,1988). Sinclair reported that plants like , *M. charantia, N. laevis* (leaves), *R. vomitoria* (leaves) and *N. laevis* (roots) that are rich in zinc and vitamin C concentration, could be effective in improving male fertility arising from zinc and vitamin C deficiency as A. senegalensis plant which is moderately rich in both zinc and vitamin C increases the testosterone production, sperm quality and motility and thus enhance male fertility (Sinclair, 2000).

The January samples of the leaves and roots of *R. vomitoria* are rich in zinc while the April samples are rich in vitamin C. Thus the January batch may be more effective than the April batch as fertility enhancers where zinc therapy is required. The leaves when processed are used to reduce swellings (Burkill, 1994). Swellings on some organs in the male anatomy may reduce fertility. The roots and leaves have been reported to contain a bioactive alkaloid which has anti prostate cancer activity (Demis *et al*., 2006). Extracts having anti-diabetic and anti-cancer activity have been obtained from the leaves, stems and roots of the plant (Chevallier, 1996). Such extracts may serve as fertility enhancers where infertility is linked to such disorders.

Thus several of the plants under investigation possess medicinal properties in addition to zinc and vitamin C and ingestion of such plant parts can enhance male fertility. *M. charantia, N. laevis* (leaves) and *R. vomitoria* (leaves) in addition to the high levels of zinc and vitamin C present have been proven to possess various components, stated previously, which can promote fertility in male subjects.

Cang *et al.*, 2008 reported that *Rv* enhance testosterone production and protect Leydig cells against oxidative stress. So also, Ajao *et al*, 2017 also reported its beneficiary effects on male wistar rat reproductive parameters (increased in testosterone concentration, sperm quality and motility) after administration of 150 mg/kg and 300 mg/kg per body weight of aqueous leaves extract of *Rauwolfia vomitoria*. More also, Ajao *et al*., 2017 reported slight slignificaant decreased in rats body weight after administration of *Rauwolfia vomitoria* leaves extract.

Figure 2.8 shown image of *Rauwolfia vomitoria* (ken *et al.,* 2014).

2.7 Reserpine

Reserpine is an indole alkaloid, antipsychotic, and antihypertensive drug that was once used to regulate high blood pressure and alleviate psychotic symptoms, but it is no longer used for these purposes due to the advent of better medications and its various side effects (Tsioufis *et al*., 2017; Hoenders *et al*., 2018). Reserpine's antihypertensive effects are due to its capacity to deplete catecholamines from peripheral sympathetic nerve endings, which has a molar mass of 608.68 g/mol. Heart rate, cardiac contraction power, and peripheral vascular resistance are all regulated by these substances (Forney, 2002).

The loss of monoamine neurotransmitters in synapses triggered by reserpine is often cited as evidence for the hypothesis that depletion of monoamine neurotransmitters induces depression in humans. This argument, however, is not without dispute. Some researchers believe that reserpine-induced depression is a myth, while others say that teas made from plant roots containing reserpine have a soothing, sedative effect that can be used as an antidepressant (Baumeister *et al*., 2003). In a randomized placebo-controlled trial, reserpine was the first

compound to be shown to be a successful antidepressant. Moreover, reserpine has a peripheral action in many parts of the body, resulting in a preponderance of the effects of the cholinergic part of the autonomous nervous system on the GI tract, smooth muscles, blood vessels and so on (Forney, 2002).

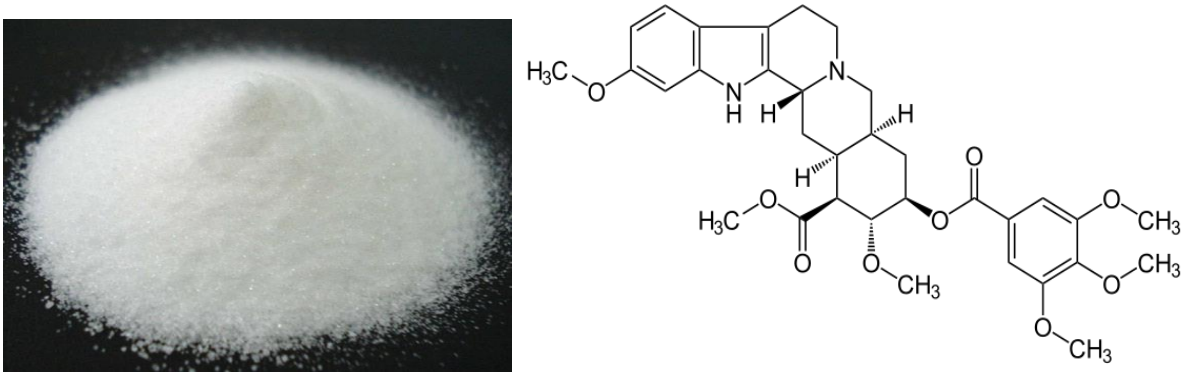


Figure 2.9 Shown image and Chemical structure of Reserpine (Forney, 2002*).*

2.7.1 Mechanism of action of Reserpine

Reserpine irreversibly blocks the vesicular monoamine transporter (VMAT) (Leão *et al*., 2017). In the presynaptic nerve terminal, this transports free intracellular norepinephrine, serotonin, and dopamine into presynaptic vesicles for release into the synaptic cleft. Unprotected neurotransmitters are metabolized in the cytoplasm by MAO and COMT, thus never excite the post-synaptic cell. Morealso, reserpine increases metabolic rate of monoamine neurotransmitters; but also decrease magnitude of monoamine release (Yaffe *et al*., 2018). It may take the body days to weeks to replenish the depleted VMAT, so reserpine's effects are long-lasting. This depletion of dopamine can lead to drug-induced Parkinsonism. Reserpine inhibits the vesicular monoamine transporter 2 (VMAT2), which prevents the absorption and storage of norepinephrine and dopamine into synaptic vesicles almost irreversibly (Eiden and Weihe, 2011).

2.7.2 Reserpine's Role in Male Reproduction

Antipsychotic drugs raise PRL, lower testosterone, and increase sexual side effects such as ejaculatory dysfunction. Chlorpromazine, haloperidol, reserpine, risperidone, and thioridazine have the most evidence, whereas aripiprazole and clozapine have less evidence. Few studies have looked at the impact of antipsychotics on sperm production, which is a major knowledge gap in reproductive pharmacology.

Psychotropic drugs are used by men of all ages and some are known to have adverse effects on reproduction in both males and females. In men, the most significant reproductive effects are on increased PRL and gonadotropin levels with decreased testosterone; sexual dysfunction, including negative effects on erection and ejaculation; and decreased semen quality. Few studies have been performed with male exposure and pregnancy or live birth as outcomes. Some psychotropics are less likely to cause adverse effects and can be substituted to improve reproductive function although well-designed, adequately powered studies are needed to confirm the value of these substitutions (Drobnis & Nangia, 2017).

Seminal vesicles removed from rats after 2 days treatment with reserpine did not contract after electrical stimulation or norepinephrine treatment, indicating blockade of α1-adrenergic receptors, which could inhibit epididymal transit and normal ejaculation (Drobnis & Nangia, 2017).

Experiments in rats have shown that reserpine treatment at HEDs for at least 2 weeks causes defects in spermatogenesis, cellular debris in the epididymides, increased epididymal fluid viscosity, decreased prostate size and lower fertility than controls (Drobnis & Nangia, 2017).

An increase in PRL has the ability to block the HPTA. The most common cause of drug-induced hyper-PRL is first-generation antipsychotic medications. The magnitude of an increase in PRL level is dose dependent and varies between drugs for a given indication. The prevalence of

hyper-PRL has been estimated to be between 23 and 72 percent in treated male patients. PRL levels increase quickly after treatment and usually return to normal within a month of stopping it. Antipsychotic drugs are known to cause elevated PRL levels in patients, according to a large body of research. Many of these articles are reviews and meta-analyses of evidence, with some examples of drugs that have no impact. When assessing the impact of psychotropics on PRL, keep in mind that bipolar mania, schizophrenia, and other psychiatric disorders may be followed by hyper-PRL, low testosterone, high LH, sexual dysfunction, ejaculatory dysfunction, and poor sperm content (Drobnis & Nangia, 2017).

A group of 62 men with schizophrenia were given haloperidol for 30 days in one trial. Their FSH, LH, and testosterone levels were low before treatment, but improved to more normal levels after treatment. In addition to endocrinopathy in schizophrenic disease, the adverse endocrine effects of psychotropic medications can be "masked" in these patients when compared to patients with other diagnoses or healthy volunteers (Drobnis & Nangia, 2017).

Reserpine has been reported to impaired spermatogenesis in the testes of pigeons Khazan *et al.,* 2007. Soad *et al.,* 2000 reported mild to severe degenerative changes in the testis of rat after acute administration of reserpine of dosage 3 mg/kg and 6 mg/kg per body weight.

2.8 Role of dopamine in the testes

Dopamine is a catecholamine that plays a role in a variety of biological processes in mammals, including cognition, emotion, and motor control, among others (Fait *et al*., 2007). The dopaminergic system and sex hormones are both essential in male sexual activity. Its cerebral control is subject to the inhibitory influence of the opioid system. A dopamine or androgen deficiency can thus cause a loss of sexual desire (Courtois & Bonierbale, 2016). High levels of catecholamine have been found in human and bovine sperm, as well as in the oviducts of rabbits, humans, pigs, and cows, at varying concentrations depending on the oviductal region and the

estrous cycle stage studied. These catecholamines are thought to come from sympathetic nerve endings that innervate both the testis and the oviduct (Javier *et al*., 2014). With respect to this, there is evidence of expression of tyrosine hydroxylase (TH), a rate-limiting enzyme in catecholamine synthesis, in the uterus and cervical cells of mare in neuronal-type cells of non-human primates and in human Leydig cells (Davidoff *et al*., 2005). This raises the possibility that catecholamines are synthesized from a source other than that of the innervation present in testis and oviduct, implying that sperm would be in contact with catecholamines, or at least with L-DOPA, a precursor of dopamine, from a very early stage during their passage through the male and female reproductive tract (Javier *et al*., 2014).

Stimulation of D2-like receptors showing high affinity for antipsychotic drugs inhibits adenylyl cyclase by coupling to inhibitory GTP-binding regulatory protein (Gi/o) and decreasing PKA activity (Missale *et al*., 1998). High concentrations of catecholamines have been detected in human semen (Fait *et al*., 2001) and in oviductal compartment from human, sow, rabbit, and cow (Kotwica *et al*., 2003). It has also been reported that catecholamine concentrations vary according to the oviductal region and during the different phases of the estrous cycle (Kotwica *et al*., 2003), suggestive of a hypothalamopituitary control system. It is likely that the source of these catecholamines is the catecholaminergic nerve terminal, although dopamine and other catecholamines are present in follicular fluid under physiological conditions (Ferna´ndez-Pardal *et al*., 1986). However, expression of tyrosine hydroxylase in equine uterus and cervical epithelia has been reported (Bae, 2001), suggesting that it is potentially available during the first stretch of the sperm journey through the female reproductive tract. In spermatozoa, many forms of neurotransmitter receptors have been discovered (Meizel, 2004), but their function is unknown. In their research, Otth *et al* observed that DRD2 expression is conserved in male germ cells and

spermatozoa (Otth *et al*., 2007). This result suggests that endogenous dopamine and antipsychotic drugs may target this receptor.

2.9 Role of CREM and Protamine Genes in Spermatogenesis

CAMP responsive element modulator is a protein that in humans is encoded by the CREM gene, (Meyer *et al.,* 1992) and it belongs to the cAMP-responsive element binding protein family. It has multiple isoforms, which act either as repressors or activators (Foulkes *et al.*,1992). CREM transcription factors plays an important role in many physiological systems, such as cardiac function, (Isoda *et al*.,2003), circadian rhythms, (Sassone-Corsi *et al*.,2000) locomotion and spermatogenesis.

cAMP responsive element seen in the promoters of many viruses and cells are usually bind to the bZIP transcription factor encodes in a CREM gene. Furthermore, CREM gene plays a key role in cAMP-mediated signal transduction during the spermatogenetic cycle and other complex processes. This gene can exert spatial and temporal specificity to cAMP responsiveness by using different promoters and translation initiation sites. This gene has several alternatively spliced transcript variants encoding several different isoforms, some of which act as transcript union activators and others as repressors. The chromosomal location of CREM gene is 10p11.21, and it starts at 35415769 and ends at 35501886 bp from pter (Sassone-Corsi *et al*., 1998).

The present discovery relates to ACT, a new protein activator of CREM in Testis, as well as the use of said protein and cDNA for screening modulating compounds that can interact with ACT or its site of interaction on CREM to block or stimulate CREM transcriptional activity in testis. These compounds have the potential to regulate male fertility. In mouse testicular tissue, an activator of cAMP-responsive element modulator (CREM) in testis (ACT) has recently been discovered. This 284-amino-acid protein belongs to the LIM-only protein family, with four complete copies of the LIM motif and one amino-terminal half. RNAse safety assays,

immunohistochemistry, and Western blot study of ACT expression in mice showed that it is only present in the testicles, especially in the nuclei of round and elongated spermatids. ACT activates CREM in a phosphorylation-independent manner, according to co-immunoprecipitation assays. ACT expression is timed to coincide with CREM production during germ cell differentiation, and the two proteins are coproduced (Fimia *et al*., 1999).

CREM regulates the transcription of a number of genes with a cAMP sensitive factor (CRE) motif in their promoter. The CREM gene produces both activator and repressor isoforms, which are expressed differently in different tissues and cells (Foulkes *et al*., 1992; Laoide *et al*., 1993) CREM has been implicated in the regulation of spermatogenesis in many studies. It regulates the expression of genes that code for structural proteins (proacrosin, protamine, RT7, mitochondrial capsule seleno protein, and others) that are necessary for spermatid differentiation into mature spermatozoa in mice (Sassine-Corsi *et al*., 1998).

Activator isoforms are only present in haploid round spermatids (stages VII and VIII) in this genus, whereas pre-meiotic germ cells only produce repressor isoforms (Foulkes *et al*., 1992). A mouse CREM deficient model demonstrates early stage spermatid arrest and azoospermia, demonstrating the significance of CREM in murine spermatogenesis (Blendy *et al*., 1996; Nantel *et al*., 1996).

Protamines are small, Arginine-rich nuclear proteins that take the place of histones late in the haploid process of spermatogenesis and are thought to be essential for sperm head condensation and DNA stabilization. The structure and compactness of chromatin in haploid spermatids undergo a number of changes during spermatogenesis (Steger, 1999). Meanwhile, transition proteins will replace the bond between deoxynucleic acid (DNA) and histone in round spermatids, while protamine will replace the transition protein in elongated spermatids. Changes in histone to protamine cause chromatin condensation in spermatozoa (Steger *et al*., 2001).

Protamine has been shown in studies to play a significant role in male fertility. Subfertile or extreme infertility is caused by PRM-1 and PRM-2 deficiency (Oliva, 2006).

FSH has been identified as a key player in the transcription of the CREM gene, and the absence of CREM-dependent transcription in post-meiotic germ cells halt spermatid differentiation and also cause programmed cell death (Sassone-Corsi, 1998). The level of CREM mRNA is primarily regulated by FSH. FSH activates Adenylate cyclase, Protein Kinase A, and cAMP-dependent PKA by binding to G-protein alpha-s -coupled receptors like the Follicle stimulating hormone receptor (FSHR). CREM gene promoters contain CRE regions, and its expression is regulated by another cAMP responsive element binding protein 1 (CREB1); CREM expression is also controlled by the autoregulatory pathway (Monaco, 1995; Hogeveen and Sassone-Coris 2006). A germ cell-specific transcriptional co-activator four and a half LIM domains 5 (ACT) interactions regulates CREM production. Kinesin family member 17 (KIF17), a germ cell-specific kinesin that controls ACT subcellular localization, regulates ACT's ability to control CREM behavior. In haploid spermatids, KIF17 colocalizes with ACT and mediates ACT transport from the nucleus to the cytoplasm at particular stages of spermatid maturation. Phosphorylation of KIF17 by PKA regulates its movement (c-AMP-dependent). Neither the motor domain nor microtubules are needed for KIF17 to shuttle between the nuclear and cytoplasmic compartments and transport ACT. (Kotaja *et al.,* 2005; Hogeveen and Sassone-Coris 2006). As a result of ACT activating CREM gene, several important genes in postmeiotic germ cells, such as PRM I and PRM II, which are both involved in DNA condensation and spermatid efficiency during spermatogenesis, are transcribed.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Animal models

A total number of seventy-two (72) adult male Wistar rats weigh between 180-200g was procured from the Central Animal House College of Health Sciences, University of Ilorin, bred and housed in the animal house of the Faculty of Basic Medical Sciences, College of Health Sciences, University of Ilorin. The animals were divided into nine groups with each group comprising eight (8) rats. The animals were caged in the animal room of the Department of Anatomy, University of Ilorin and were acclimatized for 2 weeks under standard laboratory conditions of temperature 27-30^{0}C. The animals were fed with rat pellet (TopFeeds Ltd. Nigeria) with access to drinking water ad libitum. Ethical approval with approval number (UERC/ASN/2017/1067) was gotten from University of Ilorin ethical review committee (UERC) and all procedures were done according to global best practices and institutional guidelines on the care and use of animals.

3.2 Experimental Design

Seventy-two adult male Wistar rats weigh between 180-200 g were used. The rats were divided into nine groups (A-I) with each group comprising five rats. Group A was designated as the control group which received physiological saline while rats of Groups B and C received 10 and 20 mg/kg of Chlorpromazine respectively. Groups D and E rats received 2.5 and 5 mg/kg of Reserpine respectively while Groups F and G received 150 and 300 mg/kg of Rauwolfia vomitoria leaves extract. Groups H and I received (2.5:100:5) mg/kg and (5:200:10) mg/kg of concurrent administration of Reserpine, Ascorbate and Zinc (RAZ) respectively for 56 days. Drug administrations were done orally using orogastric cannula.

3.3 Materials

3.3.1 Authentication and Ethanolic extraction of *Rauwolfia vomitoria* leaves

The leaves of *Rauwolfia vomitoria* was locally collected in a farm land in Osogbo, Osun State, Nigeria and identified in the Division of Botany, Department of Biological Sciences, University of Ilorin, Ilorin, Kwara state. Rauwolfia vomitoria leaves were air dried and the dried pieces was pulverize using an electric blender r (Blender/Miller III, model MS-223, Taiwan, China). 100 g of *Rauwolfia vomitoria* powder was then dissolved in 1 litre of ethanol. The solution was allowed to stand for twenty-four (24) hours with constant shaking for proper dissolution. The solution was then filtered with whatman filter paper so as to separate the filtrate from residue. The filtrate was oven dried to paste using an electric oven at 40°C.

3.3.1 Chlorpromazine, Reserpine, Zinc and Ascorbic acid (Vitamin C) Preparation

Chlorpromazine (>98% purity; CAS no: 69-09-0), Reserpine (>98% purity; CAS no: 50-55-5), Zinc gluconate (>98% purity; CAS no: 4468-02-4) and Ascorbic acid (>98% purity; CAS no: 50-81-7) were procured from Hefei TNJ chemical Co. Ltd China and authenticated at Pharmacy Department, University of Ilorin, Kwara State. 100 mg of Zinc and Ascorbic acid were dissolved in 100ml of distilled water while 100 mg of Chlorpromazine and reserpine were dissolved in 100 ml of dimethyl sulfoxide (DMSO). The solution was allowed to stand for some minutes with constant shaking for proper dissolution.

3.4 Procedures

3.4.1 Animal Body weights index

Body weights of the animals were taken at the beginning of the experiment and on the day of sacrifice, final body weight was taken. Body weight gained was estimated by subtracting initial body weight at the start of the experiment from the final body weight at the point of sacrificing the animals'.

3.4.2 Animal Sacrifies and Samples Collection

The animals were sacrificed on the 57th day of the experiment (so as to ensure complete spermatogenic cycle) and sacrification was done twenty-four (24) hours after the last drug(s) administration (to allow complete metabolism of the last administration) by anaesthetizing the animals with 80 mg/kg of ketamine and fix via transcardial perfusion method using 4% paraformaldehyde. The animals blood was drawn from the left ventricle prior to transcardial perfusion and appropriately dispensed into heparinized bottles for serum hormonal and biochemical analysis. Caudal epididymis and anterior end of the hypothalamus was excised prior to perfusion fixation for sperm analysis and GnRH hormone concentration from hypothalamic homogenate. Furthermore, the remaining part of the testes was fixed in 4% paraformaldehyde containing borate buffer for immunohistochemical analysis.

Diencephalon was dissected out by an anterior coronal section, anterior to the optic chiasma (Figure 3.1, AA line), and a posterior coronal cut at the posterior border of the mammillary bodies (Figure 3.1, BB line). For separation of the Arcuate (Arc) from the anteroventral periventricular (AVPV) nuclei, the third coronal cut was made in middle of the optic tract, just rostral to infundibulum (Figure 3.1, CC line). Then, rostral or anterior (contains AVPV nucleus) and caudal or posterior (contains Arc nucleus) divisions were fixed in 4% paraformaldehyde for hematoxylin and eosin histological staining.

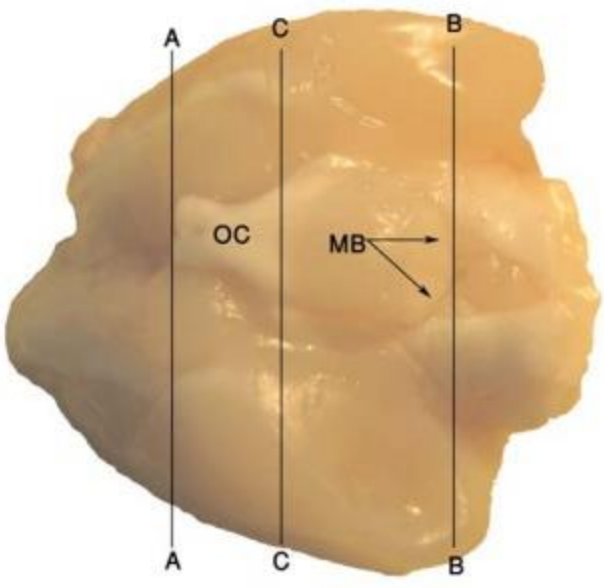


Figure 3.1: Shown separation of hypothalamic nuclei (Mohammed *et al*., 2012)

3.4.3 Reverse Transcriptase Polymerase Chain Reaction Analysis (RT-PCR)

Small testicular tissue was excised prior to perfusion fixation and put inside ependorf bottles containing trizol reagent. Testicular tissues were homogenized in cold (4^0C) TRI reagent (Zymo Research, USA, Cat: R2050-1-50, Lot: ZRC186885). Total RNA was isolated from freshly excised testicular tissues. Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges and Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70ml absolute ethanol BDH Analytical Chemicals, Poole England Cat: 10107-7Y) in 30ml of nuclease- free water (InqabaBiotec, West Africa, Lot no: 0596C320, code E476-500ML). The pellets were air dried for 5min and dissolved in RNA buffer (1mM sodium citrate, PH6.4).

Prior to cDNA conversion, total RNA quantity (concentration µg/ml) =40* A260) and quality (> 1.8) was assessed using the ratio of A260/ A280 (A= absorbance) read using spectrophotometer (

Jen-way UV-VIS spectrophotometer model 6305, UK). DNA contamination was removed from RNA was removed following DNAse 1 treatment (NEB, cat: M0303S) as specified by the manufacturer. 2µl solution containing 100 ng DNA-free RNA was converted to cDNA M-MuLV Reverse transcriptase kit (NEB, Cat: M0253S) in 20µl final volume (2µl, N9random primer mix; 0.2 µl, RNase Inhibitor (40 U/ µl) and 10.8 µl nuclease- free water). The reaction proceeded at room temperature O/N. Inactivation of M-MuLV Reverse transcriptase was performed at 65^0C/20 min.

PCR amplification for the determination of genes whose primers (SnapGene software) was done using the following protocol: PCR amplification was performed in a total of 25µl volume reaction mixture containing 2 µl cDNA (40 ng), 2 µl primer (100 pmol) 12.5µl Ready Mix Taq PCR master mix (One Taq Quick- Load 2x, master mix, NEB, Cat: M0486S) and 8.5 µl nuclease-free water. Initial denaturation at 950C 5 minutes was followed by 20 cycles of amplification (denaturation at 95^0C for 30 seconds annealing for 30 seconds and extension at 72^0C for 60 seconds) and ending with final extension at 72^0C for 10 minutes. In all experiments, negative controls were included where reaction mixture has no cDNA. The amplicons were resolved on 1.5% agarose gel (Cleaver Scientific Limited: Lot 14170811) in Tris (RGT reagent, china, Lot; 20170605)-Borate (JHD chemicals, China, Lot 20141117)-EDTA buffer (pH 8.4).

In gel amplicon bands images captured on camera were processed on Keynote platform. Gel density quantification was done using image J software. Each point represent relative expression (test gene band intensity/ internal control band intensity)* 100) plotted using Numbers software Mac OSX version).

3.4.4 Hormone Measuring Assay

Blood was collected by cardiac puncture into a heparinized bottle and serum was immediately collected by centrifugation (4000 rpm at 4°C) and stored at -20°C before the specimen was assayed. All ELISA kits (LH kit with product code: 625-300; PRL kit with product code: 725-300; FSH kit with product code: 425-300 and Testosterone kit with product code: 3725-300) used for this analysis was procured from Monobind Inc. Lake Forest, CA 92630, USA. Furthermore, GnRH hypothalamic concentration was done by homogenized hypothalamus in 400 ml of pH 7.2 phosphate buffered saline (PBS) and centrifuged at 4000 rpm/ 4^0C. The obtained supernatant was kept at -20^0C until the GnRH concentration measurement. Hypothalamic GnRH concentration was quantified by specific ELISA kits (with product code MBS762089), gotten from mybiosource inc. (Houston, USA). The absorbance in each well was read in a microplate reader (Synergy HTX multi-mode reader) purchased from BioTek company United Kingdom.

3.4.4.1 Prolactin hormone assay

Reagents: PRL calibrator – 1 ml/vial, PRL enzyme reagent – 13ml/vial, Streptavidin coated plate – 96 wells, Wash solution concentrate – 20 ml, Substrate A – 7ml/vial, Substrate B – 7 ml/vial, Stop solution – 8 ml/vial.

Test procedure

The microplates wells were formatted for each serum reference; specimen were assayed in duplicate. 0.025 ml of the appropriate serum reference was added into the assigned wells using pipette. 0.100 ml of PRL – Enzyme Reagent solution was added to all wells. Themicroplates were swirled gently for 20-30 seconds to mix and covered; incubation was done for 60 minutes at room temperature. The content of the microplate was discarded by decantation. 350 ul of wash buffer and 0.100 ml of working solution was added to all wells, incubation was done at room

temperature for 15 minutes. 0.050 ml of stop solution was added to each well and was gently mixed for 20 seconds and result was generated within 30 seconds of adding stop solution.

3.4.4.2 Follicle stimulating hormone assay

Reagents: FSH calibrator – 1 ml/vial, FSH enzyme reagent – 13 ml/vial, Streptavidin coated plate – 96 wells, Wash solution concentrate – 20 ml, Substrate A – 7 ml/vial, Substrate B – 7 ml/vial, Stop solution – 8 ml/vial.

Test procedure

The microplates wells were formatted for serum reference control; specimen were assayed in duplicate. 0.050 ml of the appropriate serum reference was added using pipette. 0.100 ml of FSH Enzyme Reagent solution was added to all wells. The microplates were swirled gently for 30 seconds to mix and covered; incubation was done for 60 minutes at room temperature. The content of the microplate was discarded by decantation. 350 ul of wash buffer and 0.100 ml of working solution was added to all wells, incubation was done at room temperature for 15 minutes. 0.050 ml of stop solution was added to each well and was gently mixed for 15-20 seconds. The absorbance in each wells was read and result was generated within 30 minutes of adding stop solution.

3.4.4.3 Luteinizing hormone assay

Reagents: LH calibrator – 1 ml/vial, LH enzyme reagent – 13ml/vial, Streptavidin coated plate – 96 wells, Wash solution concentrate – 20 ml/vial, Substrate A – 7 ml/vial, Substrate B – 7 ml/vial, Stop solution – 8 ml/vial.

Test procedure

The wells on the microplates were formatted for each serum reference, and the specimens were tested twice. A pipette was used to apply 0.05 ml of the required serum reference. All wells received 0.100 mL of LH Enzyme Reagent solution. The microplates were gently swirled for 30

seconds to mix before being covered and incubated at room temperature for 60 minutes. In each well, 0.350 mL wash buffer and 0.100 mL working solution were applied, and the wells were incubated for 15 minutes at room temperature. Every well received 0.050ml of stop solution, which was gently mixed for 15-20 seconds. Within 30 minutes of adding stop solution, the absorbance in each well was measured and a result was generated.

3.4.4.4 Testosterone hormone assay

Reagents: Testosterone calibrator – 1 ml/vial, testosterone enzyme reagent – 13ml/vial, Streptavidin coated plate – 96 wells, Wash solution concentrate – 20 ml/vial, Substrate A – 7 ml/vial, Substrate B – 7 ml/vial, Stop solution – 8ml/vial.

Test procedure

Every serum reference, control, and sample specimen were assayed twice on microplates that were well formatted. Microwell strips that were not used were returned to the aluminum container, sealed, and stored at 2-6^{0}C. In each well, 0.010 ml of the required serum reference, control, or specimen was pipetted. All wells received 0.050 ml of the functioning testosterone enzyme reagent. To mix the microplate, microplate was gently swirl for 30 seconds before applying 0.050 ml biotin reagent to each well. The microplate was gently swirled for 30 seconds to blend before being covered and incubated at room temperature for 60 minutes. Aspiration was used to clear the contents of the microplate. 0.350 mL wash buffer was applied, aspirated, and the process was repeated three times. The plates were washed by an automated plate washer. Both wells received 0.100 mL of working substrate solution. To reduce reaction time variations between wells, the reagents were added in the same order and incubated at room temperature for fifteen minutes. Each well received 0.050 mL of stop solution, which was gently mixed for 20 seconds. To reduce well imperfections in a microplate reader, the absorbance in each well was

read at 450 nm using a reference wavelength of 620-630 nm. The results were read within thirty minutes of applying the stop solutions.

3.4.4.5 GnRH hormone assay

Reagents: Micro ELISA Plate (Dismountable), Lyophilized Standard, Standard dilution buffer, Biotin-detection antibody (Concentrated), Antibody dilution buffer, HRP-Streptavidin Conjugate (SABC), SABC dilution buffer, TMB substrate, Stop solution 5ml/10ml 4°C, Wash buffer, Plate Sealer.

Test Procedure

SABC working solution and TMB substrate were equilibrated for 35 min at room temperature (37 °C). Samples and reagents dilution were properly mixed.

Standard, test sample and control (zero) wells on the pre-coated plate were set and their position were recorded respectively. Each standard and sample was measured in duplicate and the plates were washed 2 times before adding standard, sample and control (zero) wells. 50 µL of sample and Biotin-detection antibody were added to each well while the blank well was added with standard dilution buffer. 50 µL of Biotin-detection antibody working solution was immediately added to each well. The plate was gently tap so as to ensure thorough mixing and the diluents was incubated for 45 minutes at 37°C.

Each well was aspirated and wash with 350 µL of wash buffer (approximately 350µL) using automated washer for three times. After the last wash, the remaining wash buffer was removed by aspiration. The plate was inverted and patted it against thick clean absorbent paper. 100 µL of SABC working solution was then added to each well and was covered with a new plate sealer before it was later incubated for 30 minutes at 37°C. Aspiration and wash processes were repeated five more times.

90 µL of TMB Substrate was added to each well and cover with a new plate sealer and then incubated for 20 minutes at 37°C. 50 µL of stop solution was added to each well and the color turn to yellow immediately. Stop solution was added in the same order the substrate solution was added. To reduce well imperfections in a microplate reader, the absorbance in each well was read at 450 nm using a reference wavelength of 620-630 nm. The results were read thirty minutes after the stop solutions were added.

3.4.5 Biochemical Examination

Before the specimen was assayed, blood was collected via cardiac puncture into a heparinized bottle, and serum was collected via centrifugation (4000 rpm at 4°C) and stored at -20°C.

3.4.5.1 Protocol for Dopamine Assay

Extraction of Standard and Samples (Extraction Plate)

20 µL of each standard, control and sample was added into the respective wells of the extraction plate using a Pipette. 400 µL of distilled water was added to all wells except to correct differences of volumes. 1ml of Extraction Buffer was then added into each well

The plate was later covered with adhesive foil and extraction was carried out for 30 min at room temperature of 25°C on an orbital shaker set at 900 rmp. After this, 2 ml of distilled water was added into each well.

The plate was covered with a new adhesive foil and left on an orbital shaker set to 900 rmp for 5 min at room temperature of 25°C. 150 µL of extraction buffer and 50 µL of acylation regent was added into each well and mixed immediately after pipetting.

Mixture was then extracted for 20 min at 25°C (without adhesive foil) on an orbital shaker set to 600 rpm. 2 ml of distilled water was then added into each well.

The plate was covered with a new adhesive foil and left on an orbital shaker (900 rpm) for 5 min at 25°C. 300 µL of Release Buffer was added into each well and the whole mixture was then placed on an orbital shaker set to 600 rpm at 25°C for 30 min.

Preparation of Concentrated Component

The concentration component was prepared by dissolving 25ml of WASHBUF CONC with 225ml of distilled water and vigorous shaking of the mixture was done.

Preparation of COMT Enzyme Solution

The COMT enzyme solution was freshly prepared directly before use. Each kit component of lyophilized COMT was dissolved in 1.25mL of distilled water and was properly mixed. 1.25 ml of coenzyme solution was added to the mixed COMT vials followed by 1.25 ml of Enzyme Buffer and 0.40 ml COMT additive to give a final volume of 4.15ml of COMT Enzyme solution per vial.

Procedure

- 75 µL of freshly prepared COMT Enzyme solution was added into each well of the Microtiter Plate and shaked briefly.
- In each well, 100 µl of extracted standard, control, and samples were applied.
- Each well received 50 µL of Dopamine Antiserum.
- The plate was then coated with adhesive foil and incubated on an orbital shaker set to 600 rpm for 120 minutes at room temperature of 25°C.
- Each well was then filled with 200 liters of substrate solution, which was incubated for 40 minutes at 25°C (without adhesive foil) on an orbital shaker set to 600 rpm.
- The substrate reaction was then stopped by pouring 50 µL of PNPP stop solution into each well, then gently shaking the plate to mix the contents.

- The optical density was measured with a spectrophotometer at 620 nm within 60 min after pipetting of the stop solution.

Calculation of Results

The obtained OD of the standard (y-axis, linear) was plotted against their concentration (x-axis, logarithmic) using an automated method. Each signal of the standards was applied for the calculation of the standard curve .The concentrations of the kit controls and of the tissue samples in ng/mL was read directly from the corresponding standard curve.

3.4.5.2 Protocol for Superoxide Dismutase Assay

Standard Preparation

20 µL of the SOD standard was diluted with 1.98mL of sample buffer to obtain the SOD stock solution. Eight clean glass test tubes were marked A-H and different volumes of SOD stock solution and sample buffer was added to each tube.

Procedure

- 200 µL of the radical detector (assay kit) was added to 10 µL of standard tubes (A-H) per well to establish the SOD standard well.
- 200 µL of the diluted radical detector was added to 10 µL of tissue samples to establish the sample wells.
- The reactions were then initiated by adding 20 µL of diluted Xanthine oxidase to all the wells (sample wells and standard well)
- The well plates were covered with the plate cover and were carefully rocked for proper mixture to occur. The well was then incubated on a shaker for 30 minutes at room temperature and the absorbance was read at 460 nm from a spectrophotometer.

Calculations

- Each standard and sample's average absorbance were measured. The linearized rate was calculated by dividing each standard absorbance by itself as well as the absorbances of all other standards and samples.
- For a typical standard curve, a graph of the linearized SOD standard rate as a function of final SOD operation (U/ml) was plotted.
- The SOD behaviors of the samples were determined by substituting the linearized rate for each sample into the equation from the linear regression of the standard curve. One unit was described as the amount of enzyme required to dismutate the superoxide radical by 50%. The cytochrome c and xanthine oxidase couple assay were used to standardize SOD behavior.

$$\text{SOD (U/ml)} = \left[\left(\frac{\text{sample LR} - \text{Y-intercept}}{\text{Slope}}\right) \times \frac{0.23 \text{ ml}}{0.01 \text{ ml}}\right] \times \text{sample dilution}$$

3.4.5.3 Protocol for Lipid Peroxidation

Before use, all kit components and the sample were brought to room temperature at 25°C.

Normal- standard reconstituted with 1.0ml Standard Diluents', held at room temperature for 10 minutes, and gently shook (not to foam). The normal concentration in the stock solution was 6,000 ng/ml. The stock solution was first diluted to 2,000 ng/mL, and the diluted standard (2,000 ng/ml) was used as the highest standard.

Wash solution – To make 600mL of wash solution (1), 20mL of Wash solution concentrate (30) was diluted with 580 mL of distilled water.

TMB substrate- Sterilized tips were used to aspirate the necessary dose of the solution.

Assay Procedure

- The diluted standard, blank, and sample were all labeled correctly. 5 wells for standard points and 1 well for blanks were prepared. Each of the dilutions of standard, blank, and samples received 50 L into the appropriate wells. Then, immediately, 50 mL of detection reagent A was applied to each well. With the aid of a microplate shaker, the plate was gently shaken. A plate sealer was used to cover the plate, which was then incubated for 1 hour at 37°C.
- The solution was aspirated and washed in an autowasher with 350 liters of 1x wash solution for each well, then allowed to sit for 1-2 minutes. This was done three times.
- 100 µL of Detection Reagent B working solution was added to each well and was then incubated for 30 minutes at 37°C after covering it with the plate sealer.
- The aspiration/wash process was repeated for a total 5 times as conducted in step 2.
- 90 µL of substrate solution was added to each well. The solution was then covered with a new plate sealer while incubation was done for 20 minutes at 37°C.
- 50 µL of stop solution was then added to each well and measurement was then conducted at 450 nm immediately.

Calculations

The competitive inhibition enzyme immunoassay technique was used in this assay, so the assay signal amplitude and DNA concentrations in the sample are inversely related. For each normal, control, and sample, the average of the duplicate readings was determined. The log of MDA concentration was plotted on the y-axis, and absorbance on the x-axis, to construct a regular curve. The graph was used to obtain MDA readings.

3.4.5.4 Protocol for Glutathione Peroxidase

- **Standard Preparation:** 120 µL of the assay was added to 50 of co-substrate mixing in three ways
- **Control wells**: 100 µL of assay buffer was added 50 µL of co-substrate mixture and 20 µL of sample in three walls
- **Sample wells:** 100 µL of assay buffer was added to 50 µL of co-substrate mixture and 20 µL of sample in three walls
- The reaction was initiated by adding 20 µL of cumenehydroperoxide to all the wells being used.
- The plate was carefully rocked for a few seconds to ensure proper mixing and the absorbance was read once every minute at 340 nm using a spectrophotometer to obtain a 5 time point.

Calculations

1. The change in absorbance was determine (ΔA_{340}) per minute by:
    a. Plotting the absorbance values as a function of time to obtain the slope of the linear portion of the curve.
    b. Two points on the linear portion of the curve were chosen, and the changes in absorbance over time were calculated using the following equations:

$$\Delta A_{340}/\text{min} = \frac{A_{340}\,(\text{Time 2}) - A_{340}\,(\text{Time 1})}{\text{Time 2 (min)} - \text{Time 1 (min)}}$$

2. The standard or non-enzymatic wells' A340/min rate was calculated and subtracted from the sample wells' rate.

3. After that, the GPx operation was measured. The NADPH extinction co-efficient of 0.00373U/L was used to calculate the reaction rate. At 25°C, one unit is described as the amount of enzyme required to convert 1.0 mmol of NADPH to NADP+ per minute.

3.4.6 Sperm Analysis

3.4.6.1 Sperm count

Caudal end of the epididymis was minced in a buffer containing a small quantity of formaldehyde at ratio 1:5 respectively. The tip of the pipette was lowered into the hemacytometer's V-shaped groove, and a small sample was loaded into the chamber. The sample was allowed to settle for 2 minutes so that the cells would avoid drifting around the chamber and be in the same plane of view. To prevent the sample from drying out, the hemacytometer was mounted on straws inside a petri dish containing a moistened filter pap.

The hemacytometer's central counting region has 25 large squares, each of which has 16 smaller squares. To prevent double counting, cells on the lines of two sides of the large square were counted.

Since cells can be more concentrated on one side of the chamber, cell counting was performed in a non-biased manner (diagonal count). Cells were counted in 5 of the 25 broad squares, and the value obtained was then multiplied by 5 to obtain the number of cells per central counting area.

The count was done under a light microscope at ×400 magnifications and expressed as $\times 10^{\wedge 6}$/ml (Oyewopo *et al*., 2010).

Calculating Sperm Concentration

Each of the nine squares on the grid has an area of 1 square mm, including the central counting area of 25 large squares, and the cover-glass is 0.1 mm above the chamber's floor. The volume of the central counting area is therefore 0.1 mm^3 or 0.1 microliter. The number of sperm per ml of diluted sample was determined by multiplying the average number of sperm over each central

counting region by 10,000, and the result was then multiplied by the dilution factor (Oyewopo *et al*., 2010).

3.4.6.2 Sperm motility

Sperm sample was diluted in buffered saline, and 10 microliters of this sample was pipetted onto a clean, prewarmed microscope slide. A coverslip was gently lowered onto the sample (so as to avoid formation of air bubbles) and the slide was examined using a microscope with a 20x objective. At least ten widely-spaced fields were examined to provide an estimate of the percentage of motile cells (Oyewopo *et al*., 2010).

3.4.6.3 Sperm Morphology

Microscope slides and nigrosin-eosin stain was prewarmed to body temperature. A drop of stain was pipet onto the end of a slide, small droplet of sperm was then pipet next to the stain. The sperm and stain were combined by inserting the edge of another slide into the drops of stain and sperm and rocking the slide back and forth a few times. The surface of the first slide was then smeared with the second slide. Waving the slide back and forth in the air dried it quickly. With a 40x objective lens, the slide was examined under the microscope (Oyewopo *et al*., 2010).

The sperm stand out as light colored objects against a dark background produced by the nigrosin-stain stain. Normal live sperm do not take up the eosin stain and appear white, while "dead" sperm (those that have lost membrane integrity) do and appear pinkish.

Spermatozoa were counted and the percentage of abnormal sperm determined. Morphologic abnormalities of spermatozoa were categorized as

- Large, small, amorphous, vacuolated, and double heads, as well as any combination of these indicates Head defect.
- Distended/irregular/bent midpiece, a midpiece that is abnormally thin, or a mixture of these indicate Neck defect.

- Absent tails, several short, hairpin, irregular width, or coiled tails, tails with terminal droplets or some combination of these indicate Tail defect.

3.4.7 Histological examination

The testes and hypothalamus were fixed in 4% paraformaldehyde, dehydrated in increasing degrees of alcohol, cleared in xylene, and quickly dipped in molten paraffin wax before being embedded in molten paraffin wax to form a paraffin block. The tissue was then sectioned at 4μm thickness from the paraffin block using the rotary microtome. The parts were transferred to a glass slide and stained with hematoxylin and eosin after floating in a water bath at 400°C. The slides were then analyzed under a light microscope at magnifications of x100 and x400 (Oyewopo *et al.*, 2016).

3.4.8 Immunohistochemical examination

The tissue blocks from the histological samples were sectioned into 4 microns with a microtome and affixed to the slide. Positively charged coated slides are used to mount tissue sections. The slides were dried after mounting to clear any water that had become trapped underneath the segment. Following that, the slides were deparaffinized (using Xylene, 100 percent ethanol, and 95 percent ethanol) and the following washes were performed:

- Slides were washed in Xylene for 3 minutes; Xylene with 100 % ethanol for 3 minutes; 100 % ethanol for 3 minutes; 95 percent ethanol for 3 minutes; 70 % ethanol for 3 minutes and 50 % ethanol for 3 minutes and the slides were finally rinse under running cold tap water

Antigen retrieval step was followed using heat-induced epitope retrieval method.

A buffer solution of heat-induced epitope retrieval was prepared by mixing the following and adjusts pH to 6.0 using HCl.

- Sodium citrate buffer (10mM Sodium citrate, 0.05% Tween 20, pH 6.0)

- Tri-sodium citrate (dehydrate) 2.94g
- 1000 ml of distilled water

Appropriate antigen retrieval buffer was added to the pressure cooker. The pressure cooker was placed on the hot plate and turns on. Once boiling, the slides were transferred from the tap water to the pressure cooker. As soon as the cooker has reached full pressure i.e. when 3 minutes has elapsed, the hotplate was turned off and the pressure cooker was placed in an empty sink. The pressure release valve was activated and cold water was run over the cooker. Once de-pressurized, the lid was opened and cold water into the cooker for 10 minutes.

To prevent tissue drying, all incubations were carried out in a humidified chamber. Drying out at any point causes non-specific binding, resulting to deep background staining. An adequate chamber for this process is a shallow plastic box with a sealed lid and wet tissue paper in the bottom. To suit the incubation chamber, the plastic serological pipettes were cut into lengths. It was glued to the chamber's rim, with each pair's two separate pipette tubes spaced about 4.0cm apart, providing a level and elevated surface for the slides to rest on away from the wet tissue paper.

Immunostaining protocol

The slides were washed for 3 minutes in TBS PLUS 0.025% TritonX-100 with gentle agitation and were blocked in 10 % normal serum with 1%BSA in TBS for 2 hours at room temperature. The slides were then drained for a few seconds and wipe around the sections with tissue paper. Primary antibody diluted in TBS with 1% BSA was applied and was incubated overnight at 40C.

On the second day, the slides were rinsed for 3 minutes in TBS 0.025% Triton with gentle agitation. Using HRP conjugate for detection, the slides were incubated in 0.3% H202 in TBS for 15 minutes and later rinsed in TBS for 5minutes. The slides were developed with chromogen for 10 minutes at room temperature and rinsed in running tap water for 5minutes before it was

Counter-stained, dehydrated in ascending grades of ethanol, cleared and mounted (Mosadegh, 2017).

3.4.9 Germinal Cell Count

Immunohistochemical analyses of the testes were captured using Olympus binocular research microscope (Olympus, New Jersey, USA) which was connected to a 5.0 MP Amscope Camera (Amscope Inc, USA). Germ cell proliferation index, apoptotic index and sertoli cell expression were counted from captured testicular images (n=8 per group/per analysis) using physical fractionators techniques (stereological grid). The slides used for germ cell count were sectioned at the same level and for each section; the count was across the testicular profile (using cellular morphology and layer-dependent cell densities) for 8 different fields of view. The stereological evaluation of volume density of the germ cell proliferation index, apoptotic index and sertoli cell expression according to the method described by Gundersen. A restricted artificial boundary was marked on the testicle limited to boundary of the testicle. On the field was placed a counting grid with a collection of evenly spaced points. All points that hit profiles were counted, regardless of their connection to the frame.

If a profile (including its boundary) covers the upper right corner of the cross where the two lines cross, the point is called a hit. The volume density was calculated as proportion of volume by simple percentile fraction; VD (profile counted/total specimen profile x 100) (Gundersen *et al.*, 1988). Positive cells were counted and recorded and their mean with standard error of mean was plotted on bar chart for each of the groups.

3.5 Statistical Analysis

GraphPad Prism version 8.03 was used for all statistical analyses. All data were expressed as Mean±SEM while differences among groups were analyzed by one-way ANOVA. Tukey's test was used to adjust for multiple comparisons. P value <0.05 was considered to be statistically significant.

CHAPTER FOUR

4.0 RESULTS ANALYSIS

4.1 MORPHOMETRIC ANALYSIS

4.1.1 Animals' Body Weight gain

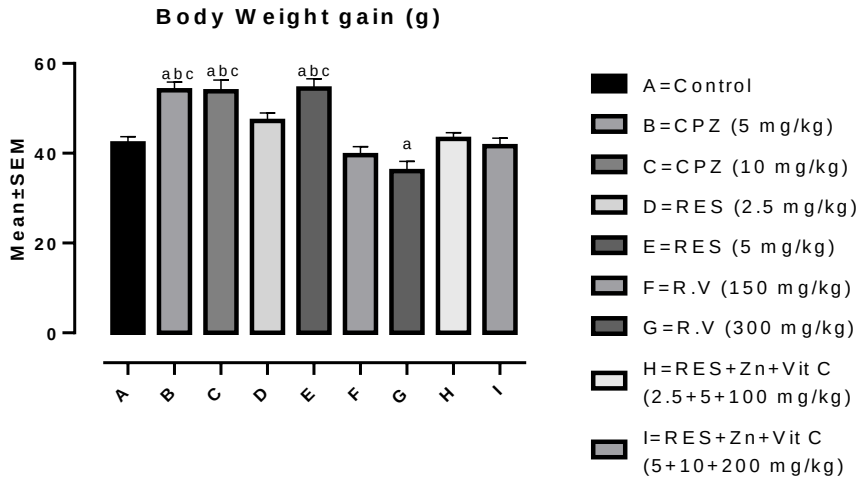


Figure 4.1.1: Showed comparison in animals' body weight gain among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. **P<0.01; (n=5).

The results depicted on figure 4.1.1 indicate the mean values outcome of the animals' body weight gained after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. From the graph, moderate significant increased were observed in 5 mg/kg, 10 mg/kg Chlorpromazine treated groups and 5 mg/kg Reserpine treated group when compared with the control group A and Co-administration of Reserpine, Ascorbate and Zinc treated groups. Furthermore, no significant difference was observed when 2.5 mg/kg of Reserpine, 150 mg/kg of *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc treated groups were compared with the control group A rats. Slight significant decresased in body weight of rats' treated with 300 mg/kg of *Rauwolfia vomitoria* was observed when compared with control group A rats'.

4.2 REVERSE TRANSCRIPTASE-PCR GENE ANALYSIS

4.2.1 RT-PCR CREM Gene Result

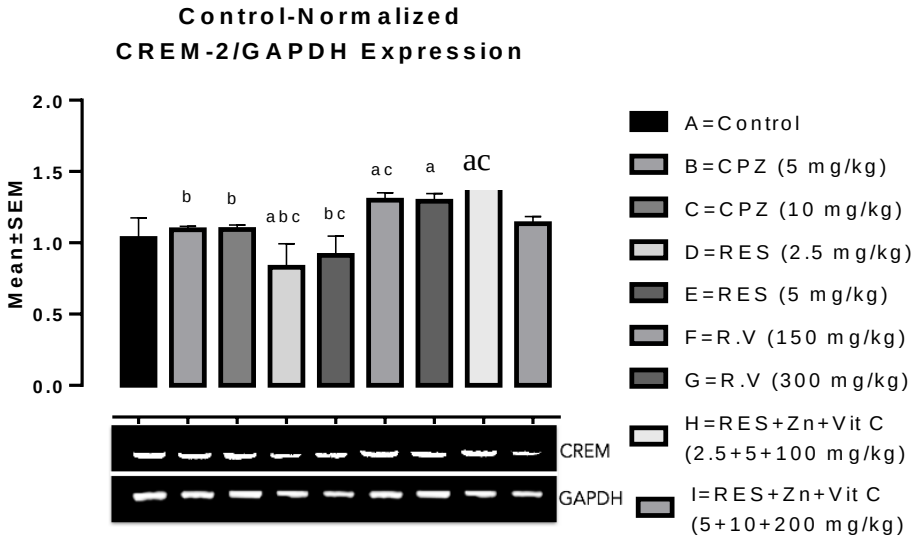


Figure 4.2.1: Showed comparison in CREM gene expression among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. **P<0.01; ***P<0.001 (n=5).

Result from figure 4.2.1 revealed CREM gene expression mean values after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. High significant upregulation of CREM gene was observed in *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups (Groups F, G and H) when compared with control group A rat. Moreover, significant down regulation of CREM gene expression was observed in 2.5 mg/kg Reserpine treated group when compared with control group A rats.

Comparison in CREM gene expression among Chlorpromazine, Reserpine and Co-administration of Reserpine, Ascorbate and Zinc treated groups showed high significant downregulation of CREM gene expression in Chlorpromazine and Reserpine treated groups (Groups B, C, D, and E) when compared with co-administration of 2.5 mg/kg Reserpine, 100

mg/kg Ascorbate and 5 mg/kg Zinc treated group H. Furthermore, Significant down regulation of CREM gene were observed in 2.5 and 5 mg/kg Reserpine treated groups D and E while significant upregulation were seen in 150 mg/kg *Rauwolfia vomitoria* and co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H when compared with co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I.

4.2.2 RT-PCR PRM-1 Gene Result

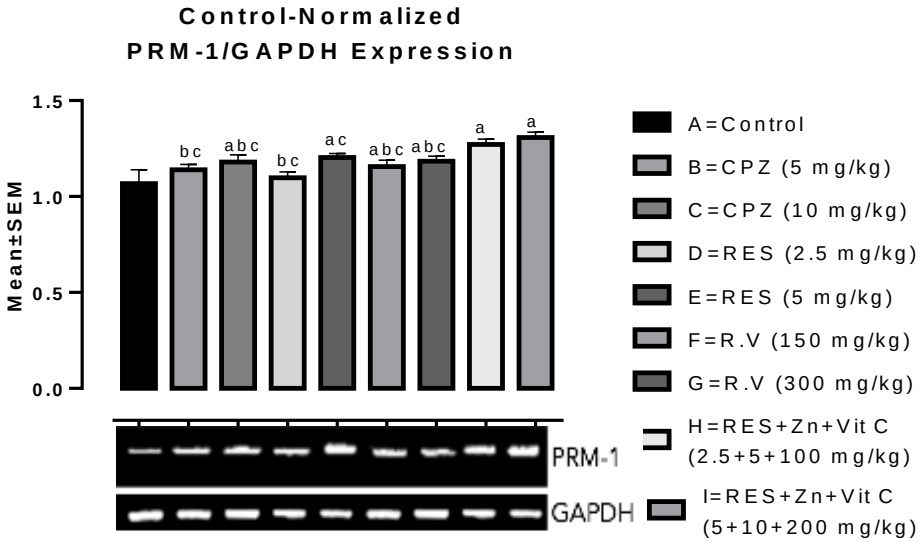


Figure 4.2.2: Showed comparison in PRM-1 gene expression among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Mean values of Protamine 1 gene expression among experimental groups has depicted in figure 4.2.2 showed high significant upregulation of PRM-1 gene in 10 mg/kg Chlorpromazine, 5 mg/kg Reserpine, 300 mg/kg *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups when compared with the control group A rats. More also, slight significant upregulation was observed in 150 mg/kg Rauwolfia vomitoriatreated group when compared with the control group A.

High significant downregulation of PRM-1 gene expression were observed in 5, 10 mg/kg Chlorpromazine, 2.5 mg/kg Reserpine and 150 mg/kg *Rauwolfia vomitoria* treated groups when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H. Moreover, slight significant downregulation of PRM-1 gene was noticed in 300 mg/kg *Rauwolfia vomitoria* treated groups when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H.

Furthermore, Chlorpromazine, Reserpine and *Rauwolfia vomitoria* treated groups B, C, D, E, F and G showed high significant downregulation of PRM-1 gene expression when compared with co-administration of 5 mg/kg Reserpine, 200mg/kg Ascorbate and 10mg/kg Zinc treated group I.

4.2.3 RT-PCR PRM-2 gene Result

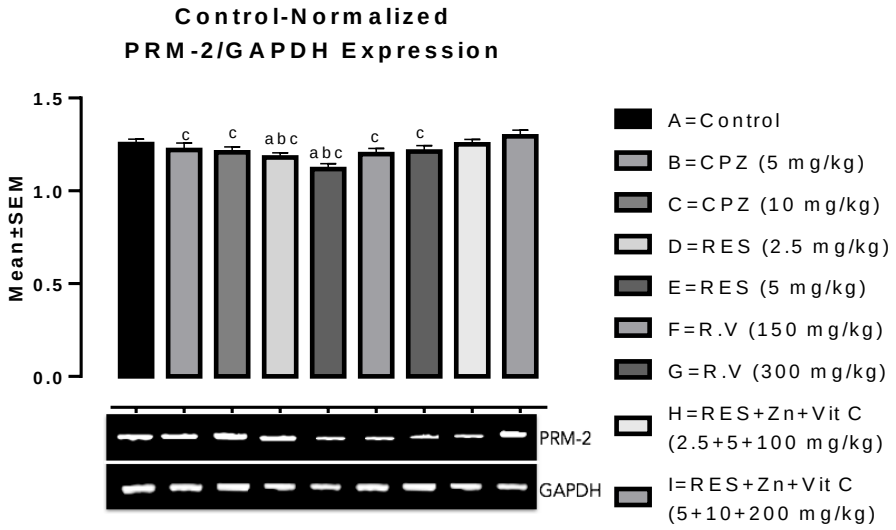


Figure 4.2.3: Showed comparison in PRM-2 gene expression among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Mean values of Protamine-2 gene expression has shown in figure 4.2.3 revealed slight significant downregulation of PRM-2 gene expression in 2.5 mg/kg Reserpine treated group when compared with control group A and co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H. In addition to this, high significant downregulation of PRM-2 gene

expression was observed in 5 mg/kg Reserpine treated group when compared with control group A and co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H.

Comparison of PRM-2 gene expression between co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I and other treated groups showed significant downregulation of PRM-2 gene expression in 5, 10 mg/kg Chlorpromazine and 300 mg/kg *Rauwolfia vomitoria* treated groups. Furthermore, high significant downregulation of PRM-2 gene expression were seen in 2.5, 5 mg/kg Reserpine and 150 mg/kg *Rauwolfia vomitoria*treated groups.

4.3 HORMONAL ANALYSIS

4.3.1 Serum Follicle Stimulating Hormone Concentration

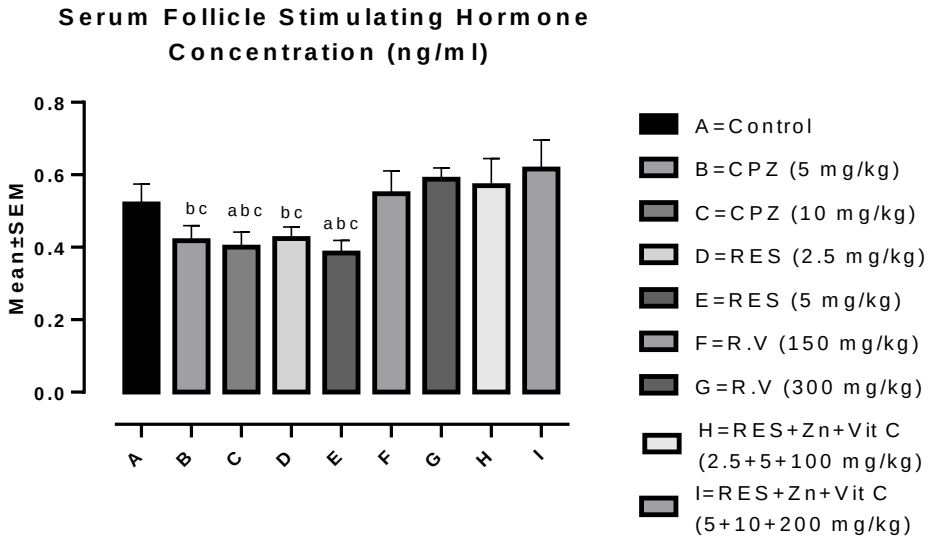


Figure 4.3.1: Showed comparison in Serum FSH Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Result from figure 4.3.1 showed mean values for serum follicle stimulating hormone concentration after administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. Slight significant decrease in serum FSH concentration was observed in 10 mg/kg Chlorpromazine treated group while moderate significant decreased was observed in 5mg/kg of Reserpine treated group when compared with the control group A.

Serum FSH concentration was significantly decreased in 5 mg/kg and 2.5 mg/kg Chlorpromazine and Reserpine respectively when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H. Furthermore, high significant decreased in serum FSH were noticed in 10 mg/kg Chlorpromazine and 5 mg/kg Reserpine when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated

group H. Comparison among co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I with synthetic antipsychotic drugs groups B (5 mg/kg Chlorpromazine), C (10 mg/kg Chlorpromazine), D (2.5 mg/kg Reserpine) and E (5 mg/kg Reserpine) showed high significant decreased in their serum FSH concentration when compared with group I.

4.3.2 Serum Luteinizing Hormone Concentration

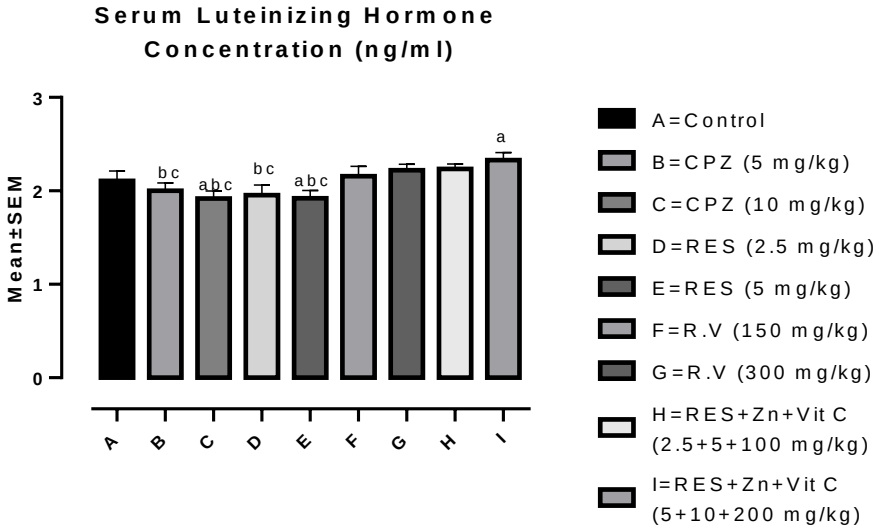


Figure 4.3.2: Showed comparison in Serum LH Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

The results on figure 4.3.2 indicate the mean values outcome of the serum luteinizing hormone after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc. From the graph, slight significant decreased in serum LH concentration were seen in 10 mg/kg Chlorpromazine and 5 mg/kg Reserpine while significant increased was observed in co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups when compared with control group A.

Comparison among co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group Aand co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate

and 10 mg/kg Zinc treated group I with synthetic antipsychotic drugs groups B (5 mg/kg Chlorpromazine), C (10 mg/kg Chlorpromazine), D (2.5 mg/kg Reserpine) and E (5 mg/kg Reserpine) revealed high significant decreased in serum LH concentration in all synthetic antipsychotic drugs treated groups.

4.3.3 Serum Testosterone Hormone Concentration

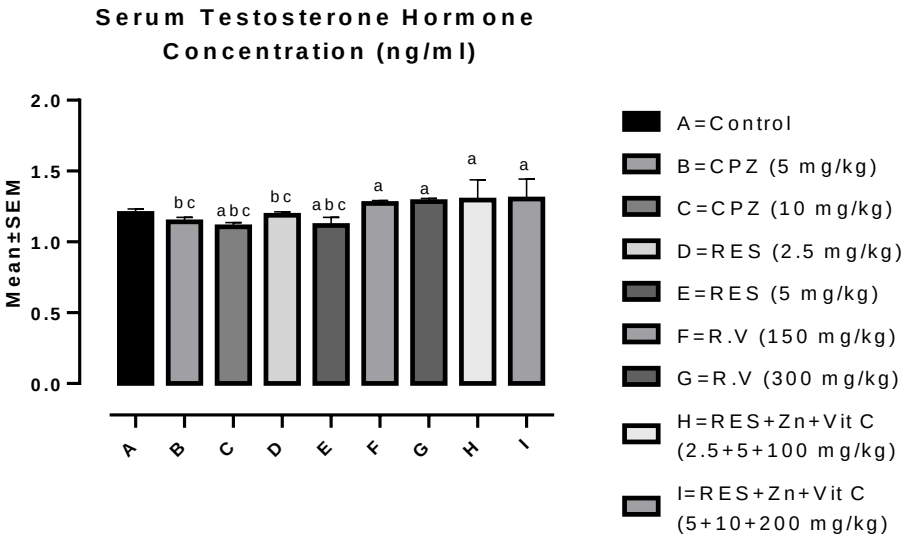


Figure 4.3.3: Showed comparison in Serum Testosterone Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Figure 4.3.3 graph revealed mean values of serum testosterone concentration among the groups after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. Serum testosterone were significantly decreased in 10 mg/kg Chlorpromazine and 5 mg/kg Reserpine treated groups while high significant increased was observed in 300 mg/kg *Rauwolfia vomitoria*, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups when compared with control group A.

Moreover, comparison among co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group A and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I with synthetic antipsychotic drugs groups B (5 mg/kg Chlorpromazine), C (10 mg/kg Chlorpromazine), D (2.5 mg/kg Reserpine) and E (5 mg/kg Reserpine) revealed high significant decreased in serum LH concentration in all synthetic antipsychotic drugs treated groups.

4.3.4 Serum Prolactin Hormone Concentration

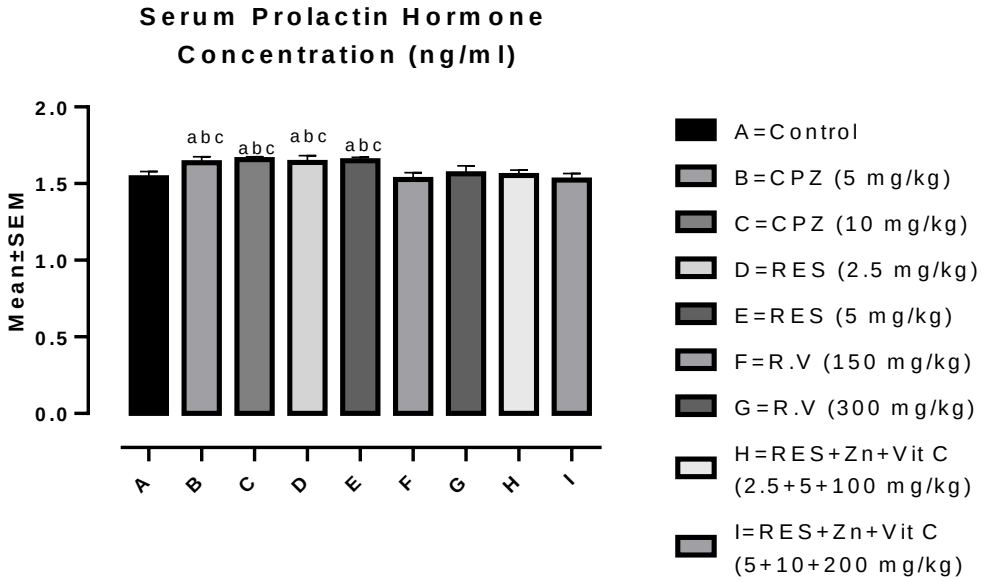


Figure 4.3.4: Showed comparison in Serum Prolactin Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Prolactin mean values as depicted in figure 4.3.4 revealed high significant increased in serum prolactin concentration in synthetic antipsychotic treated groups B (5 mg/kg Chlorpromazine), C (10 mg/kg Chlorpromazine), D (2.5 mg/kg Reserpine) and E (5 mg/kg Reserpine) when compared with the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups. More also, no significant difference was observed among

control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

4.3.5 Hypothalamic GnRH Concentration

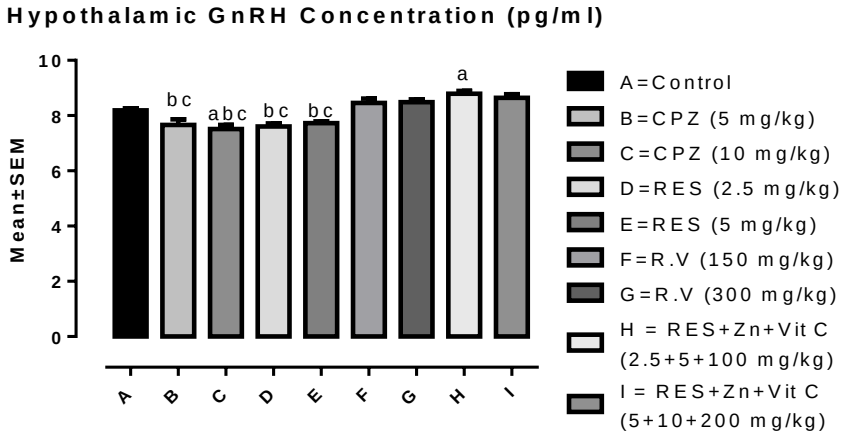


Figure 4.3.5: Showed comparison in hypothalamic GnRH Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Mean values of hypothalamic GnRH concentration has shown in figure 4.3.5 revealed slight significant decreased in 10 mg/kg Chlorpromazine treated group and slight significant increased in co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group when compared with control group A. Moreover, high significant decreased in hypothalamic GnRH concentration was observed in 5 mg/kg Chlorpromazine, 10 mg/kg Chlorpromazine, 2.5 mg/kg Reserpine and 5 mg/kg Reserpine treated groups when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I. No significant difference noticed in *Rauwolfia vomitoria* treated groups when

compared with the control and Co-administration of Reserpine, Ascorbate and Zinc treated groups.

4.4 BIOCHEMICAL ANALYSIS

4.4.1 Serum Malondialdehyde Concentration

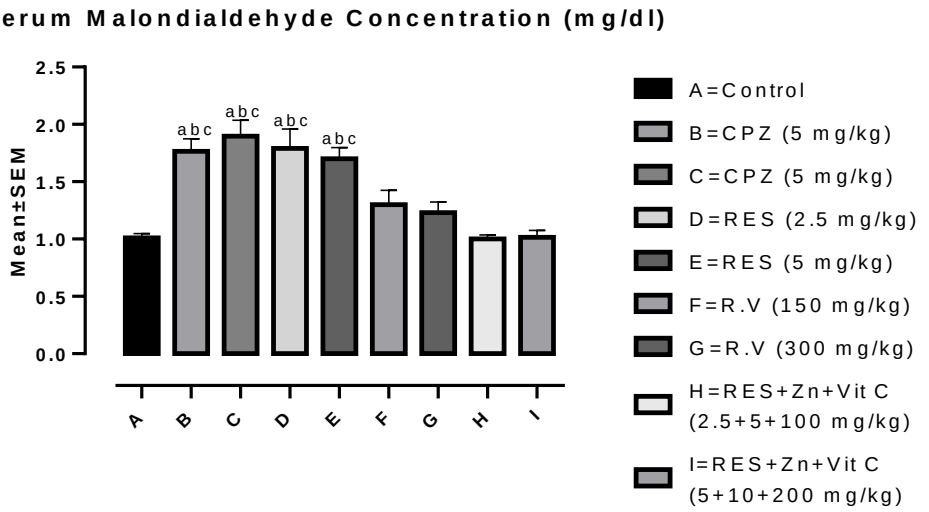


Figure 4.4.1: Showed comparison in Serum Malondialdehyde Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

As revealed in figure 4.4.1, high significant increased in serum malondialdehyde concentration in synthetic antipsychotic treated groups B (5 mg/kg Chlorpromazine), C (10 mg/kg Chlorpromazine), D (2.5 mg/kg Reserpine) and E (5 mg/kg Reserpine) when compared with the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups. More also, no significant difference was observed among control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

4.4.2 Serum Superoxide Dismutase Concentration

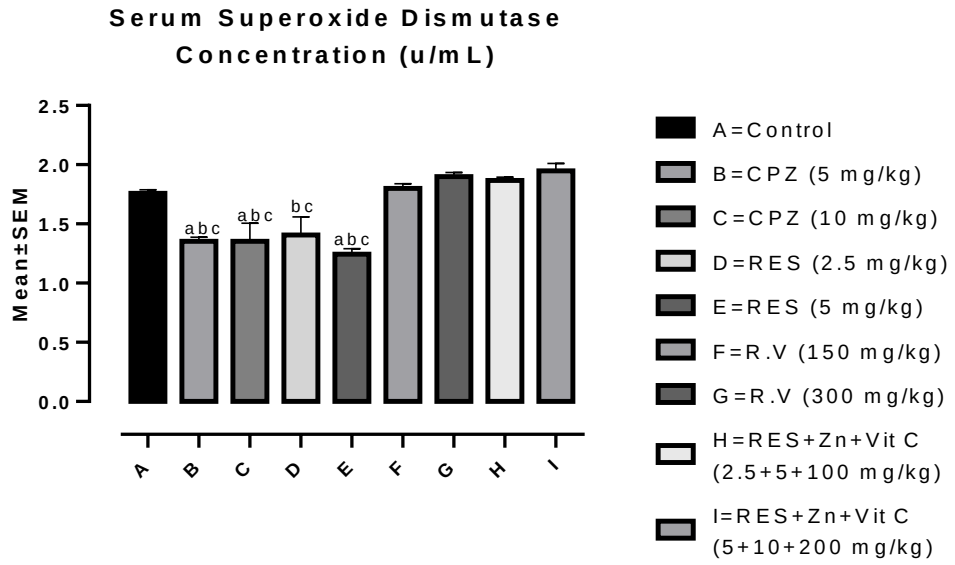


Figure 4.4.2: Showed comparison in Serum Superoxide Dismutase Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Figure 4.4.2 Showed comparison in Serum Superoxide DismutaseConcentration among the groups after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. Slight significant decrease was observed in Chlorpromazine treated groups when compared with control group A while moderate significant decreased was seen in Chlorpromazine and Reserpine treated groups when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H. Furthermore, high significant decreased was noticed in Chlorpromazine and Reserpine treated groups when compared with co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I. No significant difference was observed among control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

4.4.3 Serum Glutathione Peroxidase Concentration

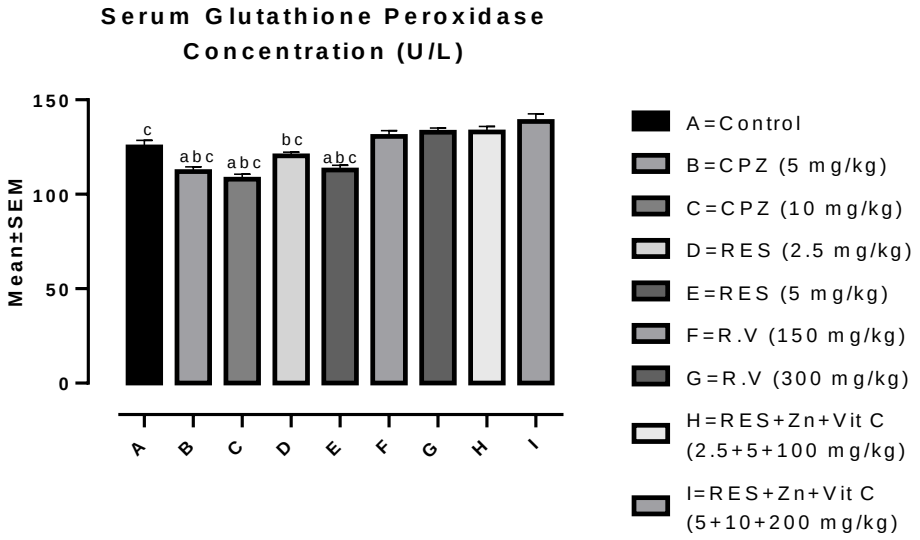


Figure 4.4.3: Showed comparison in Serum Glutathione Peroxidase Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Mean values of Serum Glutathione Peroxidase Concentration as shown in figure 4.4.3 revealed slight significant decreased in 5 mg/kg Chlorpromazine and 5 mg/kg Reserpine treated groups while high significant decreased was observed in 10 mg/kg when compared with control group A rats. No significant difference was seen among the control group A, 2.5 mg/kg Reserpine and co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated groups. Furthermore, high significant decreased was noticed in serum GPx concentration when co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups were compared with Chlorpromazine and Reserpine treated groups. In addition, slight significant increased in serum GPx concentration was seen in co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group when compared with control group A.

4.4.4 Serum Dopamine Concentration

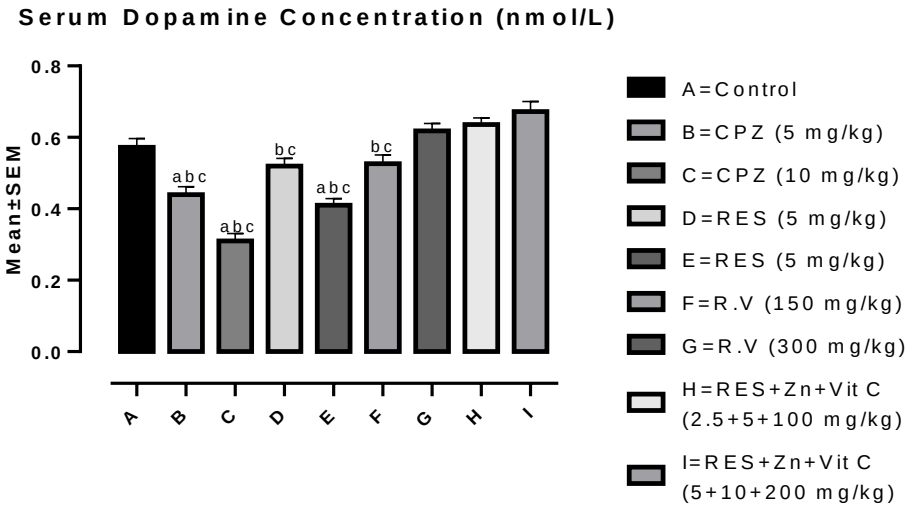


Figure 4.4.4: Showed comparison in Serum Dopamine Concentration among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group **A; b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Figure 4.4.4 showed mean values of serum dopamine concentration after administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. From the graph, high significant decreased in serum dopamine concentration was observed when 5 mg/kg Chlorpromazine, 10 mg/kg Chlorpromazine and 5 mg/kg Reserpine treated groups were compared with control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

Furthermore, slight significant decreased was seen in 2.5 mg/kg reserpine and 150 mg/kg *Rauwolfia vomitoria* treated groups when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc group H while high significant decreased was noticed when 2.5 mg/kg reserpine and 150 mg/kg *Rauwolfia vomitoria* treated groups was compared with co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc group I rats.

4.5 SPERM ANALYSIS

4.5.1 Total Sperm Count Grading

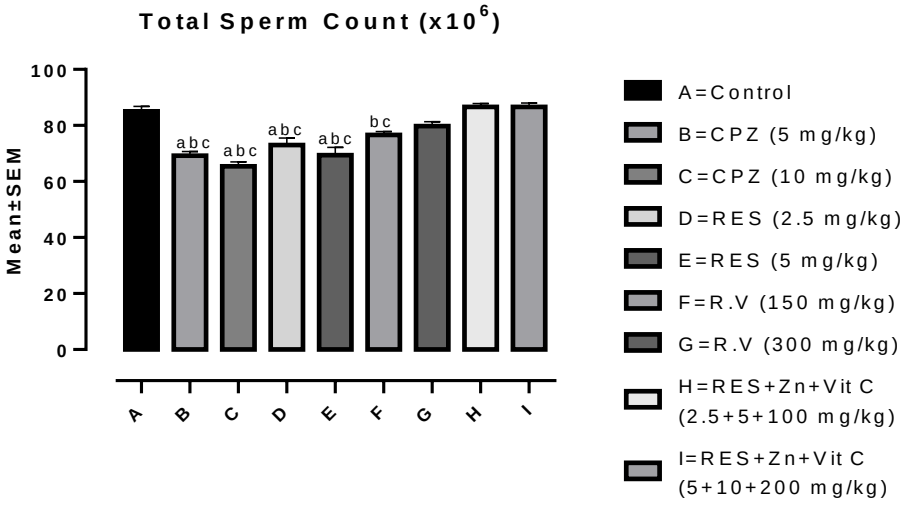


Figure 4.5.1: Showed comparison in total sperm count grading among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Total sperm count grading as depicted in figure 4.5.1 revealed high significant decreased in total sperm count among Chlorpromazine and Reserpine treated groups when compared with control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups. Moreover, no significant difference was seen among the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

Slight significant decreased in total sperm count was observed when 150 mg/kg *Rauwolfia vomitoria* treated group was compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

4.5.2 Normal Sperm Morphology Grading

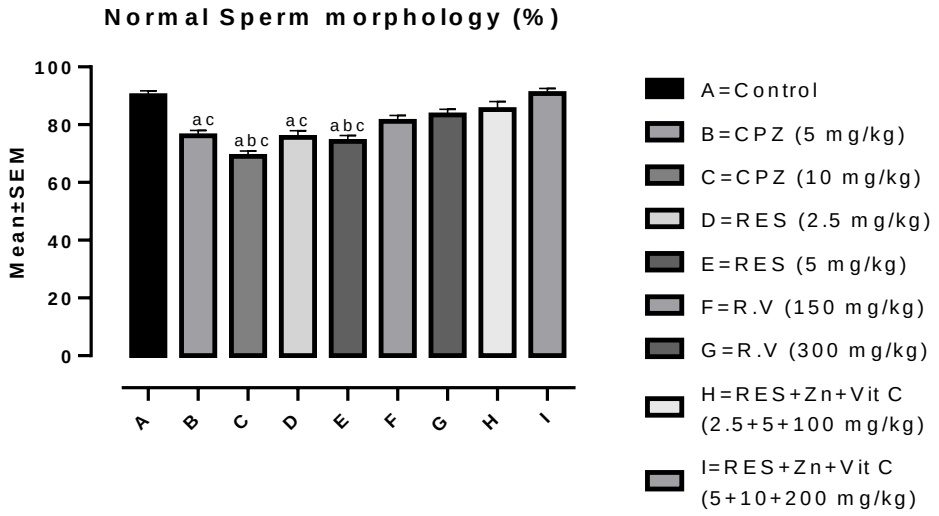


Figure 4.5.2: Showed comparison in normal sperm morphology among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I.*P<0.05; **P<0.01; ***P<0.001 (n=5).

Result from figure 4.5.2 revealed high significant decreased in normal sperm morpholohy among Chlorpromazine and Reserpine treated groups when compared with control group A and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups. Moreover, no significant difference was seen among the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

10 mg/kg Chlorpromazine treated group showed high significant reduction in normal sperm morphology while 5 mg/kg reserpine treated group showed slight significant reduction when compared with co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc treated group H.

4.5.3 Motile Sperm Grading

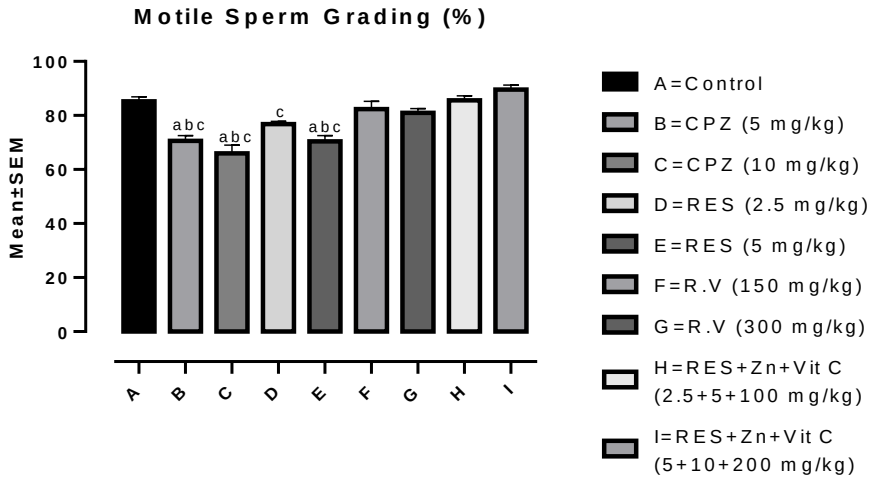
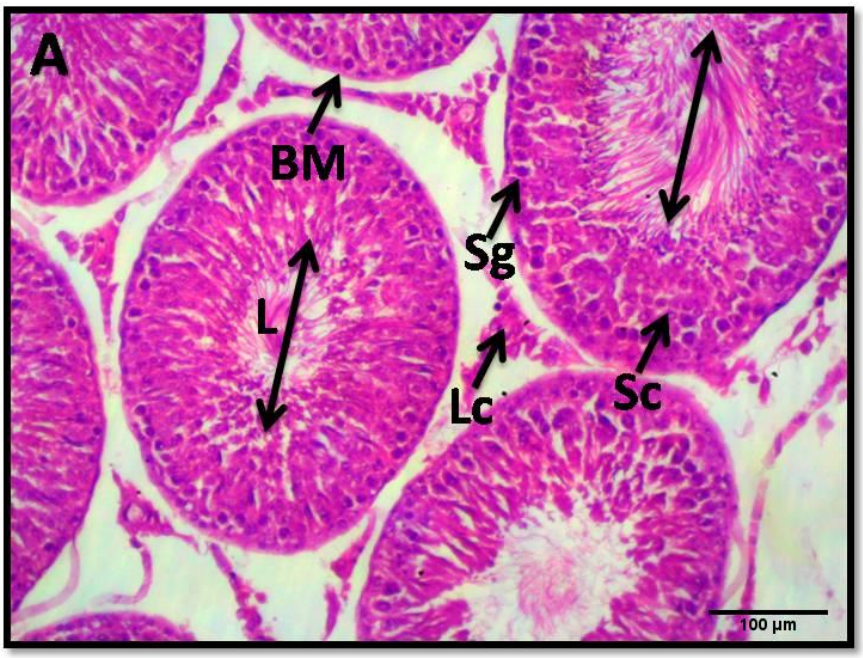


Figure 4.5.3: Showed comparison in Motile Sperm Grading among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. **P<0.01; ***P<0.001 (n=5).

Motile sperm grading mean values as depicted in figure 4.5.3 showed high significant reduction in the motile sperm among 5 mg/kg Chlorpromazine, 10 mg/kg Chlorpromazine and 5 mg/kg reserpine treated groups when compared with the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups. More also, slight significant decreased was noticed in 2.5 mg/kg reserpine treated group when compared with and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated group I. no significant difference was seen among the control group A, co-administration of 2.5 mg/kg Reserpine, 100 mg/kg Ascorbate and 5 mg/kg Zinc and co-administration of 5 mg/kg Reserpine, 200 mg/kg Ascorbate and 10 mg/kg Zinc treated groups.

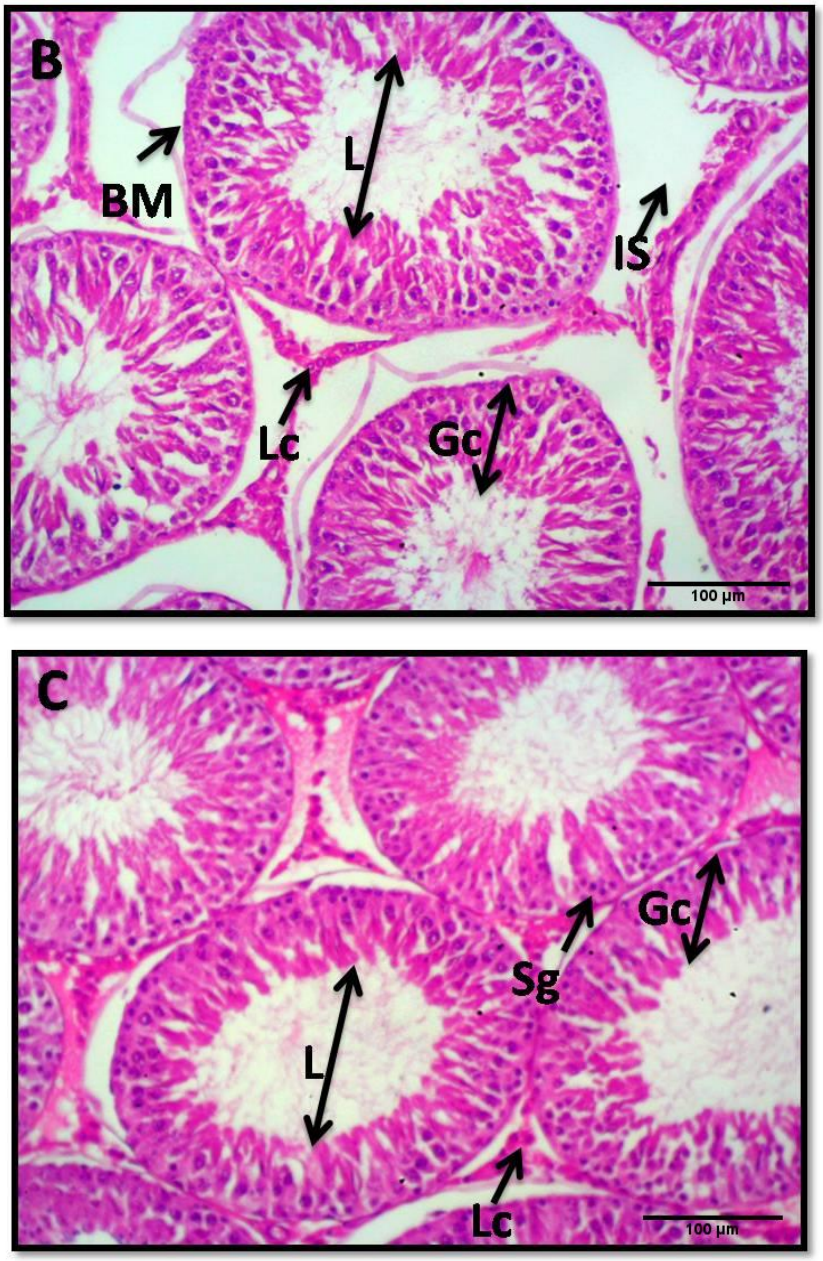
4.6 HISTOLOGICAL EXAMINATION OF TESTES AND HYPOTHALAMUS

4.6.1 Testes Stained with Hematoxylin and Eosin dye



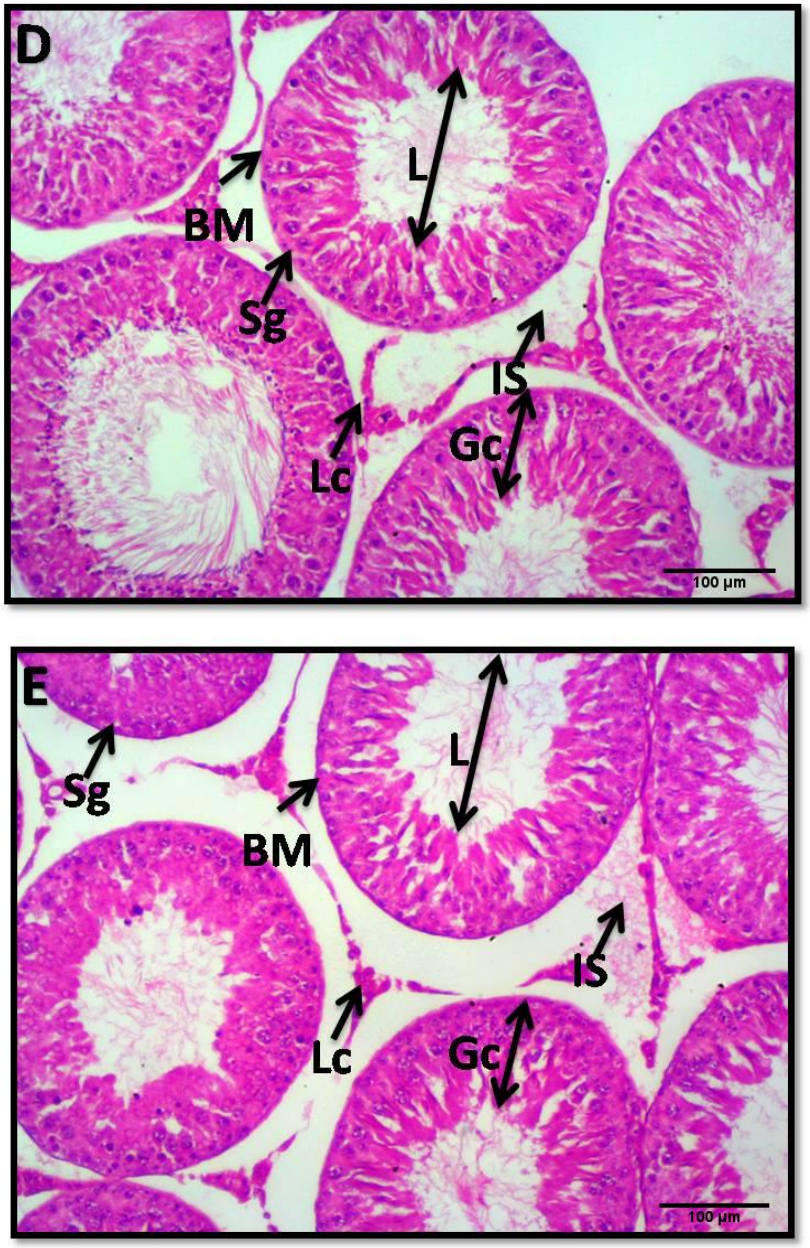
Photomicrographs cytoarchitectural presentations of Control group A testicular slide stained with Hematoxylin and Eosin dyes, (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are basement membrane (BM), lumen (L), leydig cells (Lc) sertoli cells (Sc) and spermatogonia cells (Sg).

Figure 4.6.1 showed photomicrograph of control group A animals which revealed normal testicular architecture without any observable presentation of spermatogenic arrest and the lumen could also be observed with the presence of spermatozoa. The basement membrane is thin and the interstitial space contains leydig cells.



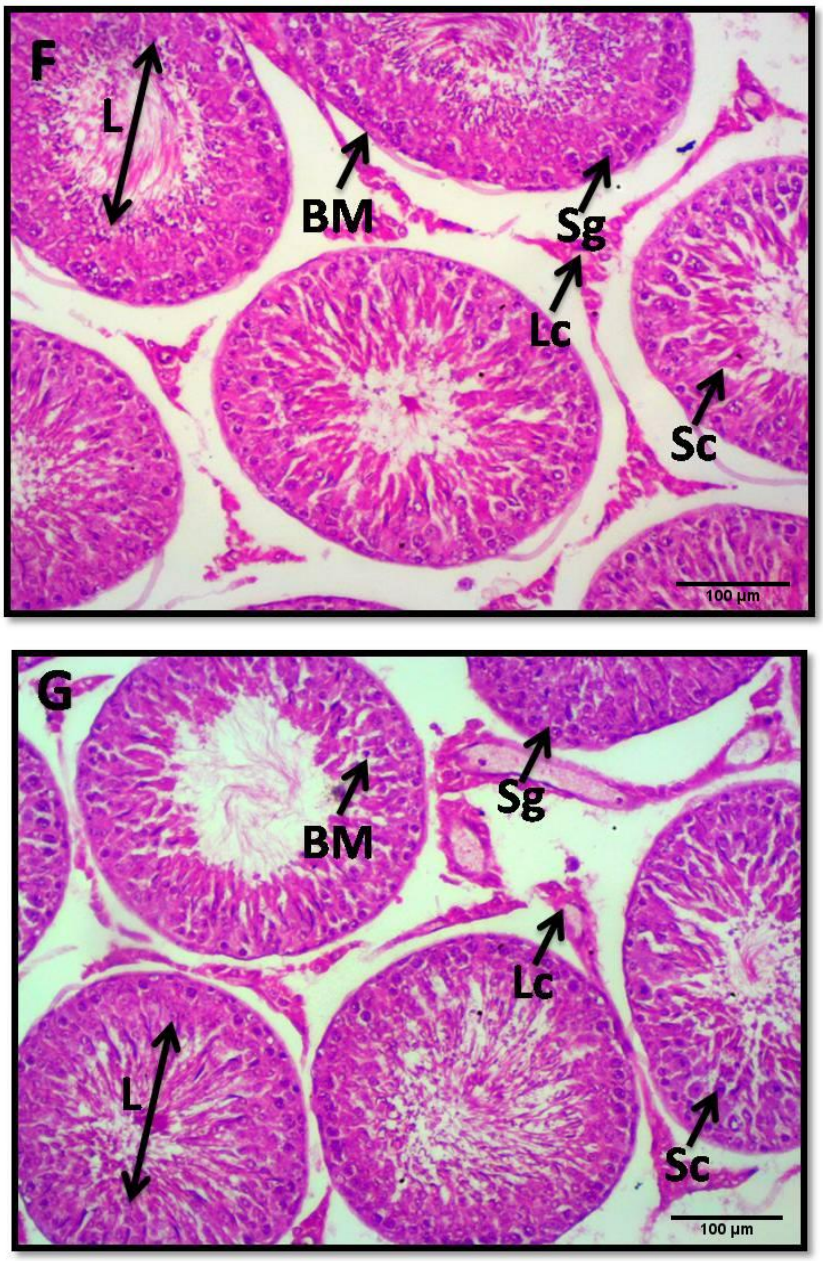
Photomicrographs cytoarchitectural presentations of groups B and C testicular slide stained with Hematoxylin and Eosin dyes, (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

Figure 4.6.1 showed photomicrograph of Groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively) severe observable degenerative changes characterized by maturation arrest of the spermatogenic cell line in several seminiferous tubules, widened lumen that lack spermatozoa (black thin arrow), fragmented basement membrane and pyknotic leydig cells.



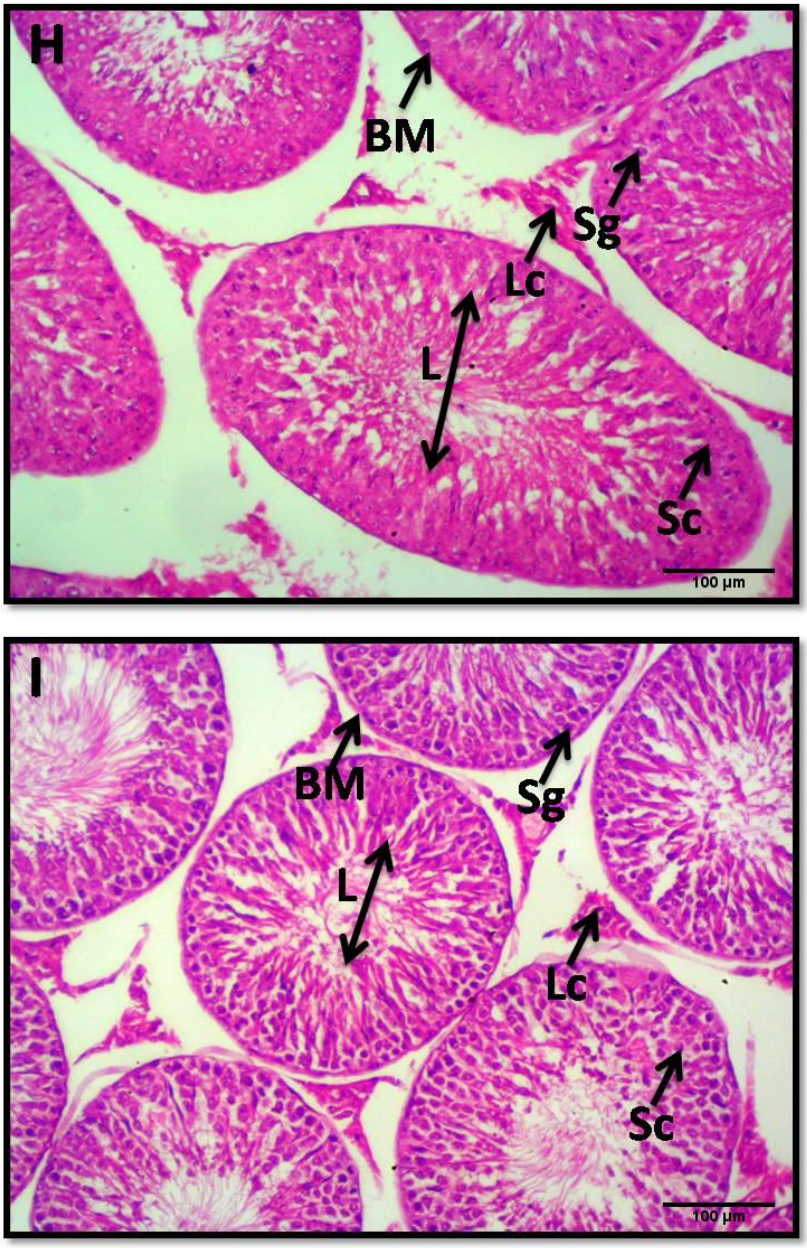
Photomicrographs cytoarchitectural presentations of groups D and E testicular slide stained with Hematoxylin and Eosin dyes, (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are germinal cell (Gc), basement membrane (BM), lumen (L), leydig cells (Lc), interstitial space (IS) and spermatogonia cells (Sg).

Figure 4.6.1 showed photomicrograph of Groups D and E treated with RES (2.5 mg/kg and 5 mg/kg respectively) mild degenerative changes characterized mainly by wide lumen with little spermatozoa in some seminiferous tubules was observed in Group D while severe degenerative changes characterized mainly by wide lumen with no spermatozoa in most of the semeiniferous tubules.



Photomicrographs cytoarchitectural presentations of groups F and G testicular slide stained with Hematoxylin and Eosin dyes, (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are basement membrane (BM), lumen (L), leydig cells (Lc), sertoli cells (Sc) and spermatogonia cells (Sg).

Figure 4.6.1 showed photomicrograph of Groups F and G treated with RV (150 mg/kg and 300 mg/kg respectively) testicular slides appeared normal and was characterized by normal differentiation of spermatogenic cell lines, presence of leydig cells in the interstitial spaces and the presence of spermatozoa in the lumen.

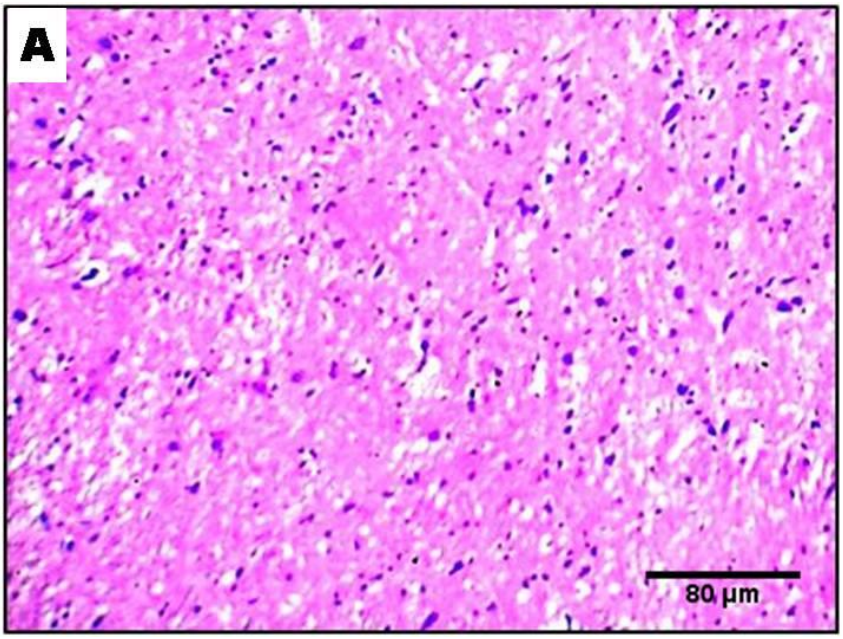


Photomicrographs cytoarchitectural presentations of groups H and I testicular slide stained with Hematoxylin and Eosin dyes, (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are basement membrane (BM), lumen (L), leydig cells (Lc), sertoli cells (Sc) and spermatogonia cells (Sg).

Figure 4.6.1 showed photomicrograph of Groups H and I treated with RAZ (2.5:5:100 mg/kg and 5:10:200 mg/kg respectively) similar morphological presentation with similar staining intensity and cellular density were observed in groups F and G when compared with the control group. The testicular cytoarchitecture was well structured and characterized by seminiferous tubules

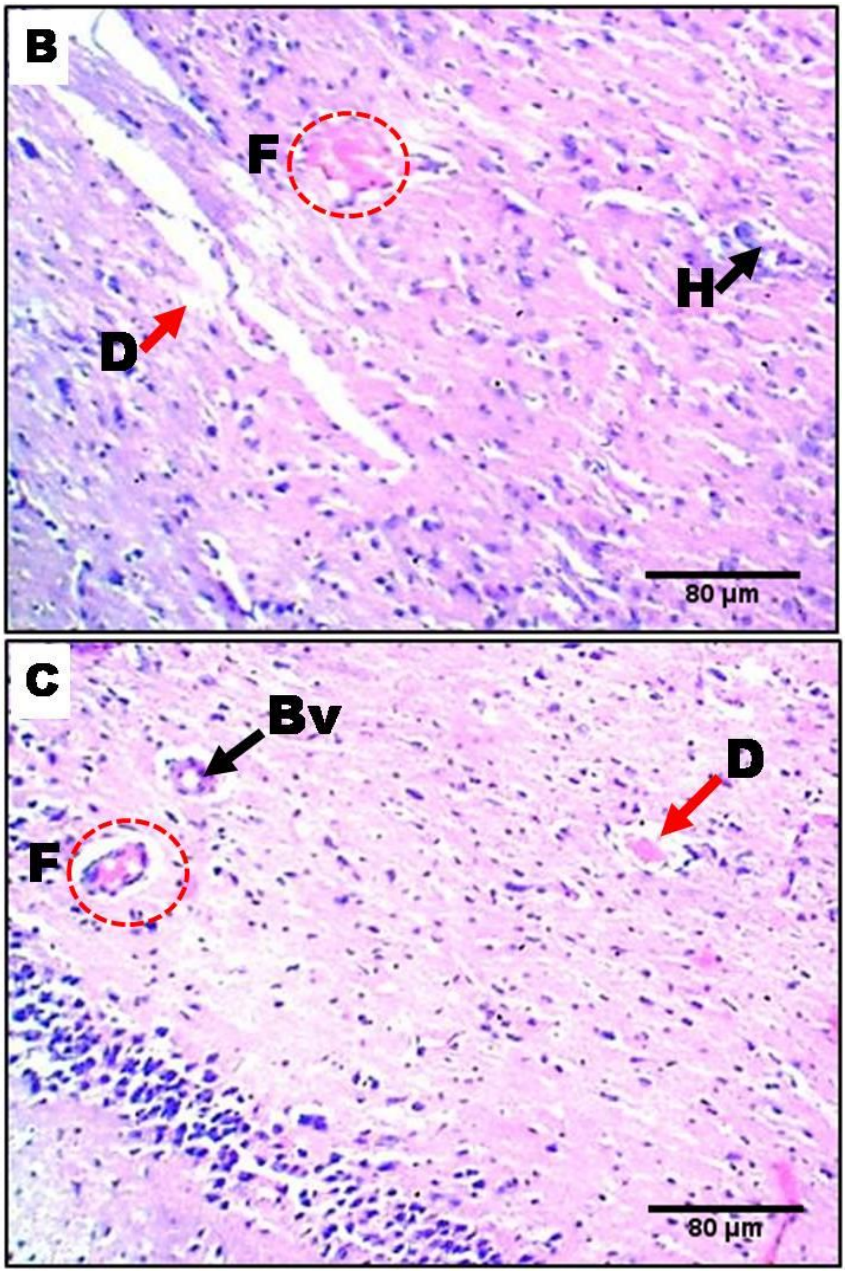
having numerous Spermatogonia cells that have differentiated into numerous Spermatocytes, the presence of sertolic cells at the adlumina border, presence of leydig cells in the interstitial spaces, and seminiferous tubule lumen filled with spermatozoa.

4.6.2 Hypothalamus Stained with Hematoxylin and Eosin dye



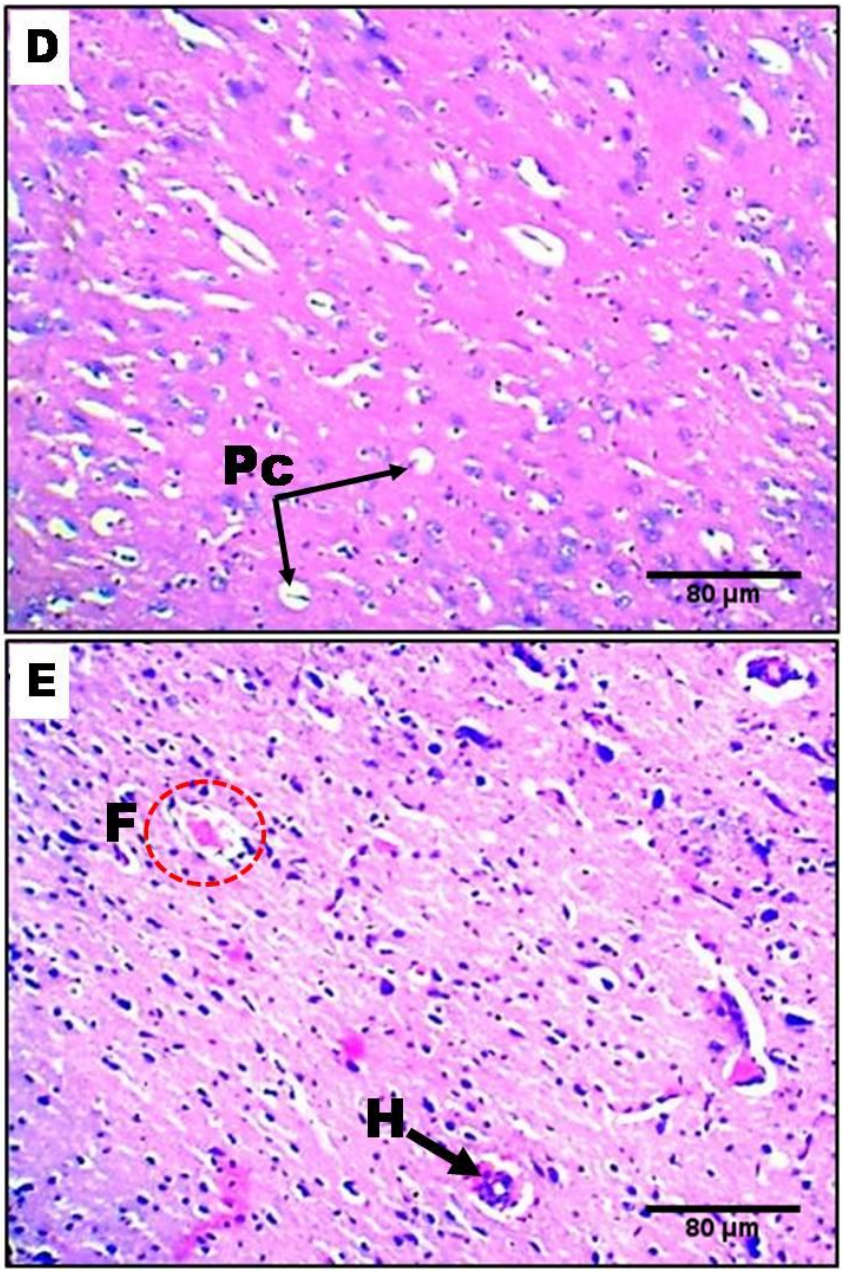
Photomicrographs cytoarchitectural presentations of control group A hypothalamic slide stained with Hematoxylin and Eosin dyes, (Scale bar: 80 µm).

Figure 4.6.2 showed photomicrograph of control group A that received physiological saline present with numerous neurosecretary. Morealso, no observable cell death, pyknosis or hamartoma in the control group A rats.



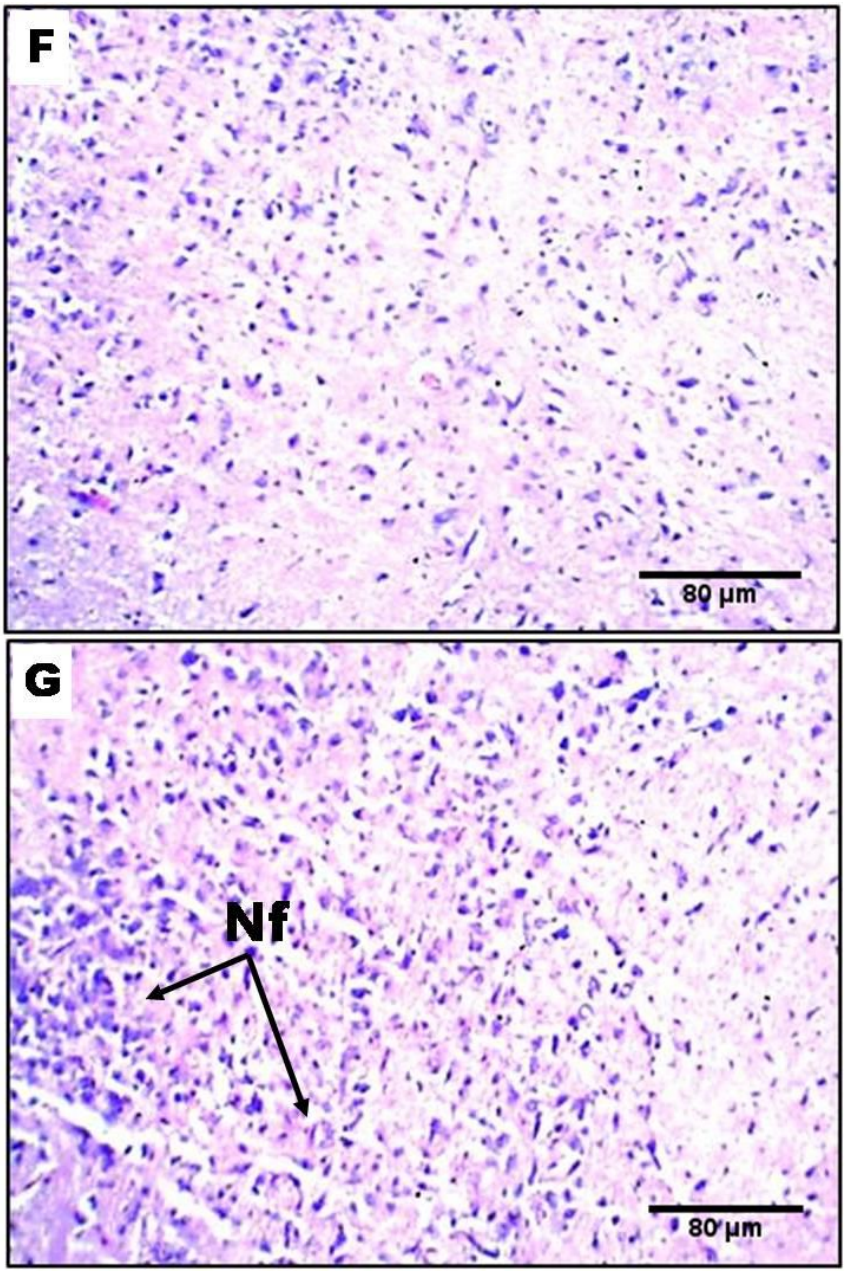
Photomicrographs cytoarchitectural presentations of groups B and C hypothalamic slide stained with Hematoxylin and Eosin dyes, (Scale bar: 80 µm). Presented in the slides are (Bv) Blood vessel, (F) Fibrosis, (Pc) Pyknotic cells and (D) area with distortions.

Figure 4.6.2 showed photomicrograph of groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively) with severe loss of hypothalamic neurosecretory cells, presence of red inflammatory cells (Fibrosis), reduced cellular density and some non-cancerous growths (Hamartoma).



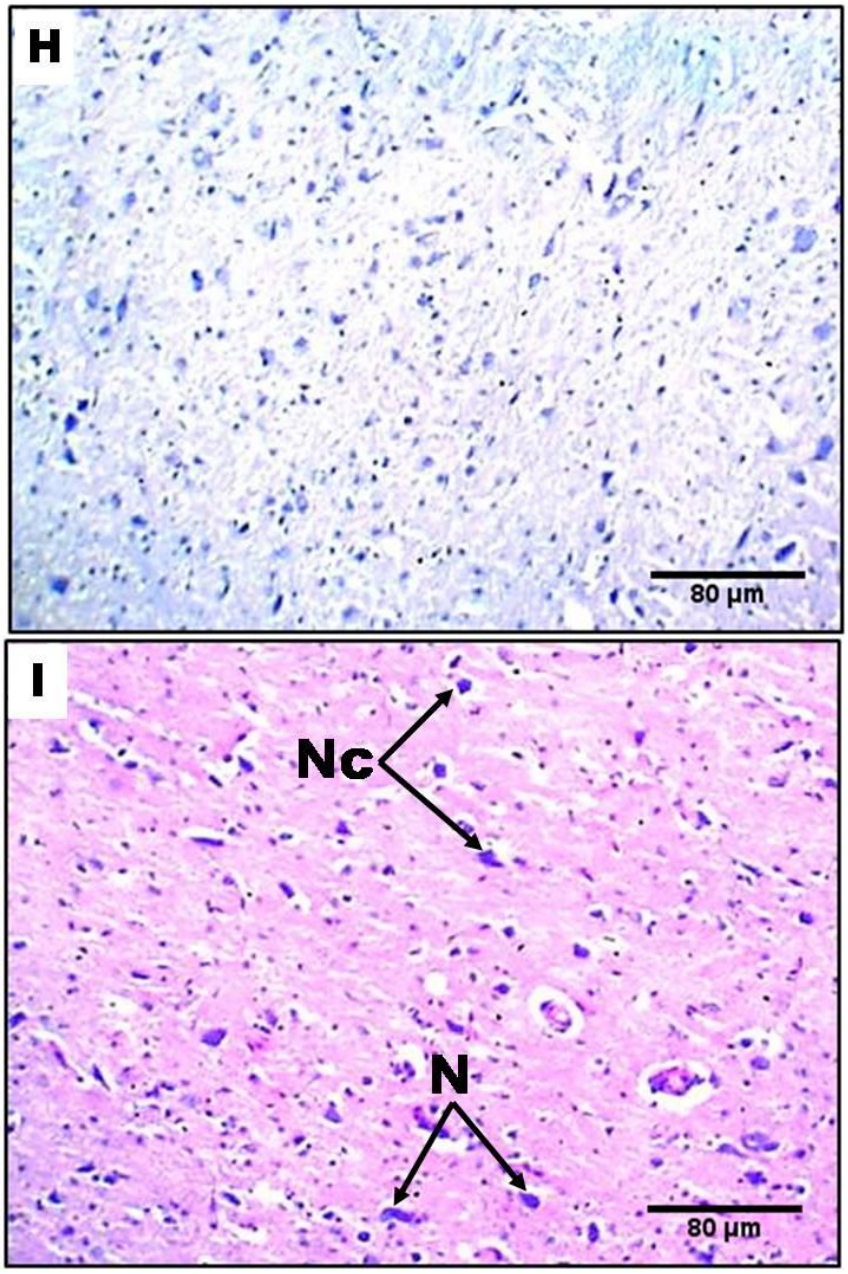
Photomicrographs cytoarchitectural presentations of groups D and E hypothalamic slide stained with Hematoxylin and Eosin dyes, (Scale bar: 80 µm). Presented in the slides are (Bv) Blood vessel, (F) Fibrosis, (Pc) Pyknotic cells and (D) area with distortions.

Figure 4.6.2 showed photomicrograph of groups D and E treated with RES (2.5 mg/kg and 5 mg/kg respectively) with condensation and reduction in hypothalamic nuclei (Pyknosis), presence of red inflammatory cells (Fibrosis) and some non-cancerous growths (Hamartoma).



Photomicrographs cytoarchitectural presentations of groups F and G hypothalamic slide stained with Hematoxylin and Eosin dyes, (Scale bar: 80 µm). Presented in the slides is (Nc) Neurosecretory cells.

Figure 4.6.2 showed photomicrograph of groups F and G treated with RV (150 mg/kg and 300 mg/kg respectively) with numerous neurosecretory cells, increased cellular density and good cellular layering.

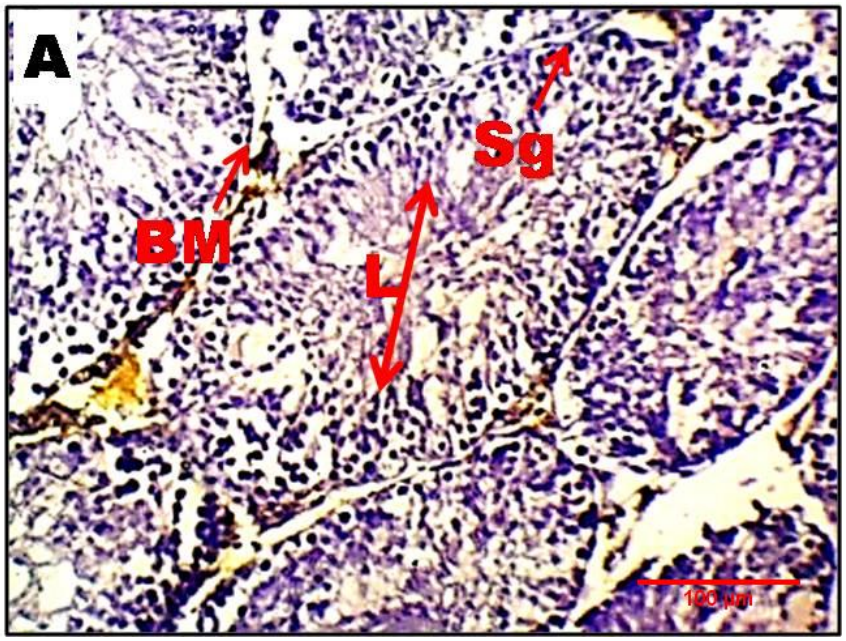


Photomicrographs cytoarchitectural presentations of groups H and I hypothalamic slide stained with Hematoxylin and Eosin dyes, (Scale bar: 80 µm). Presented in the slides are (Nc) neurosecretory cells and (N) neurons.

Figure 4.6.2 showed photomicrograph of groups H and I treated with RAZ (2.5:5:100 mg/kg and 5:10:200 mg/kg respectively). Groups H and I had similar presentation with control group A with numerous neurosecretory cells, increased cellular density and good cellular layering when compared with CPZ and RES treated groups.

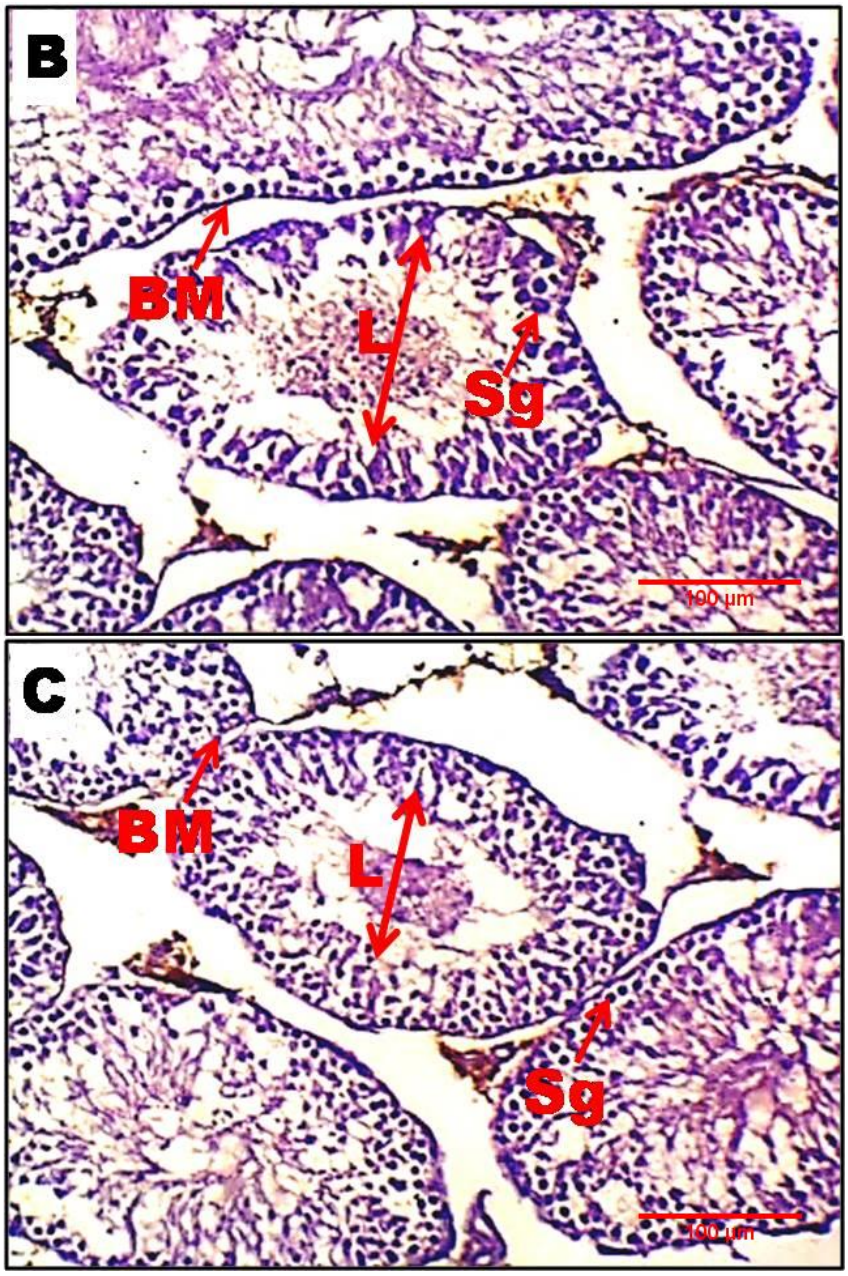
4.7 IMMUNOHISTOLOGICAL EXAMINATION OF TESTES

4.7.1 Ki-67 Protein expression in Rats' Testes



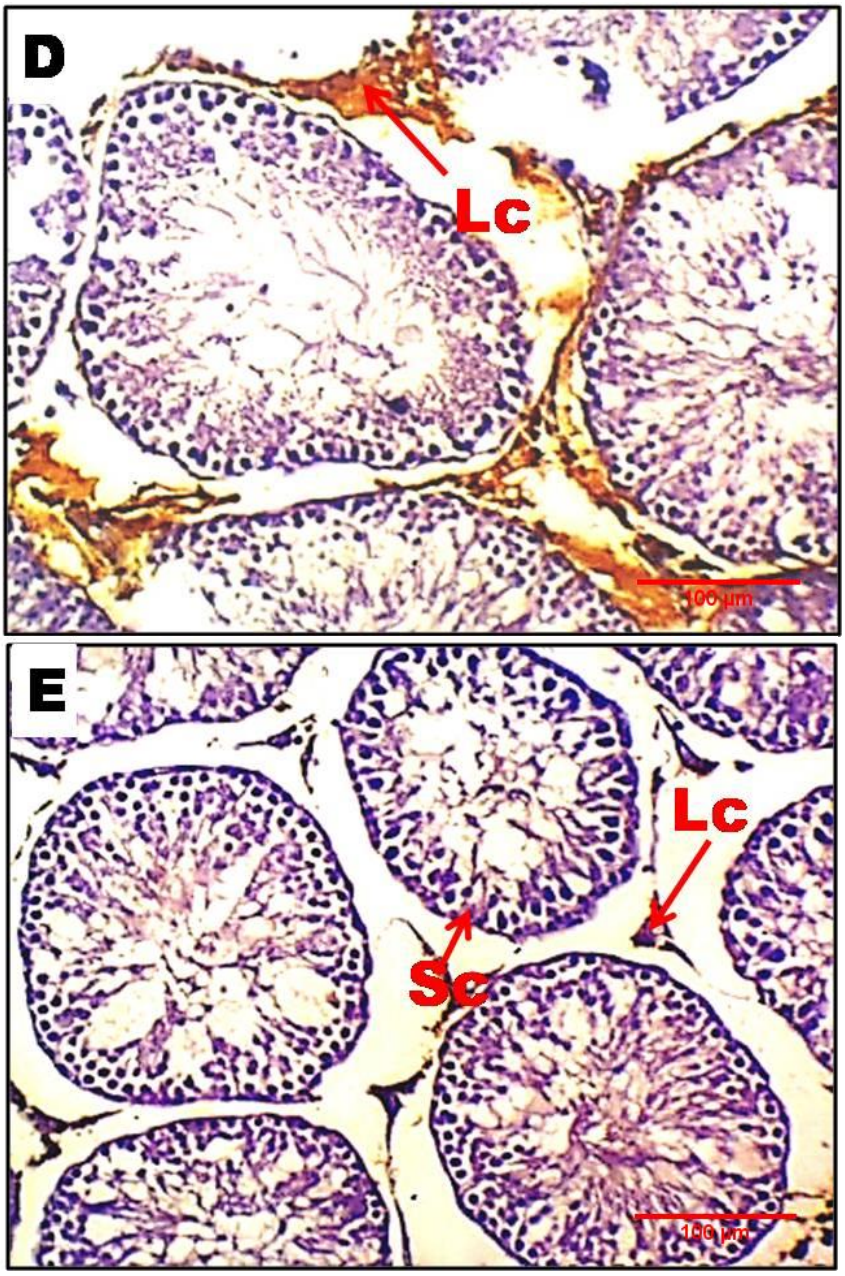
Immunohistochemical staining for Ki-67 in control group A (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the basement membrane (BM), lumen (L) and spermatogonia cells (Sg).

Figure 4.7.1 depicts immunohistochemical staining for Ki-67 in control group A with strong positive Ki-67 immunoreactivity which indicates normal proliferation and differentiation of spermatogenic cell lines.



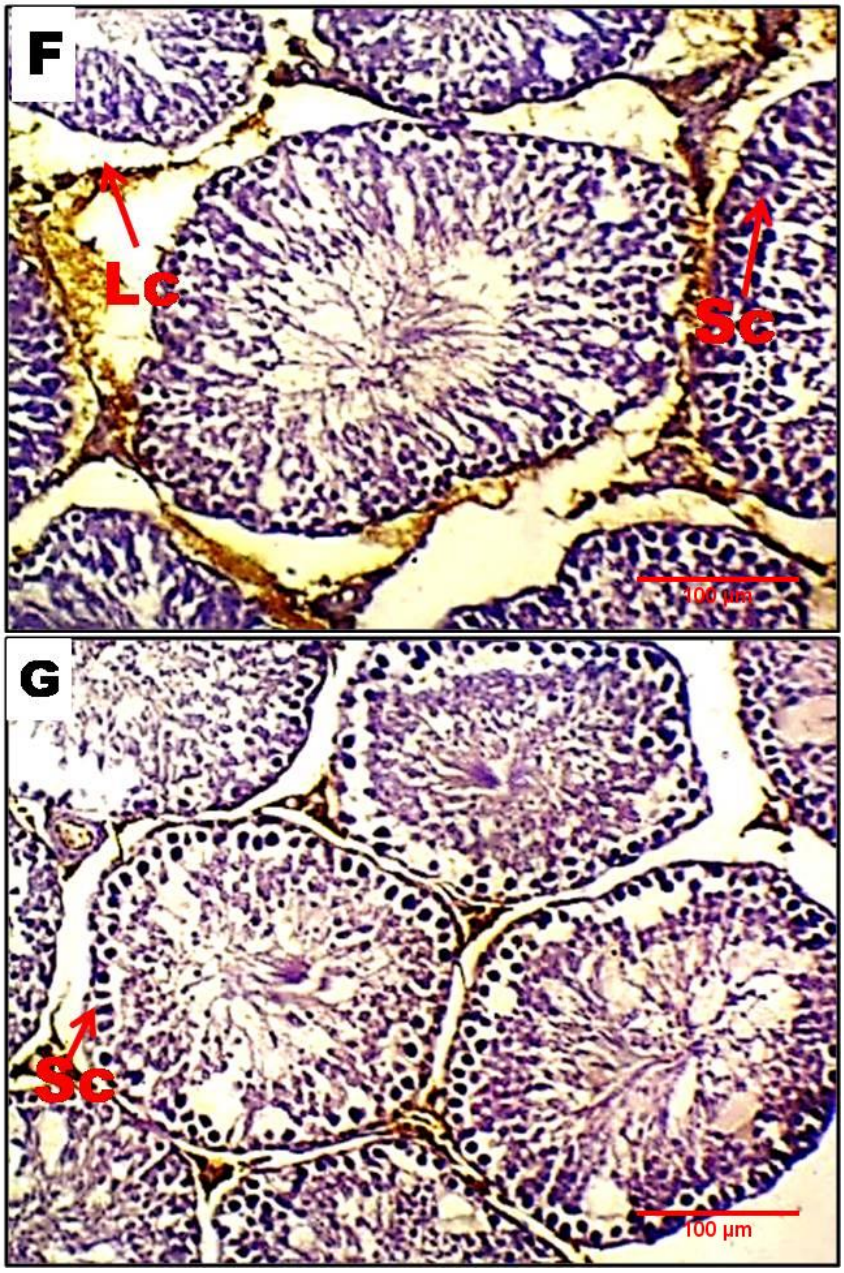
Immunohistochemical staining for Ki-67 in groups B and C (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the basement membrane (BM), lumen (L) and spermatogonia cells (Sg).

Figure 4.7.1 depicts immunohistochemical staining for Ki-67 in groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively). Both groups B and C showed weak Ki-67 immunoreactivity, presented with lightly stained dark brown granules which signal poor proliferation and arrest of differentiation in testicular germ cells.



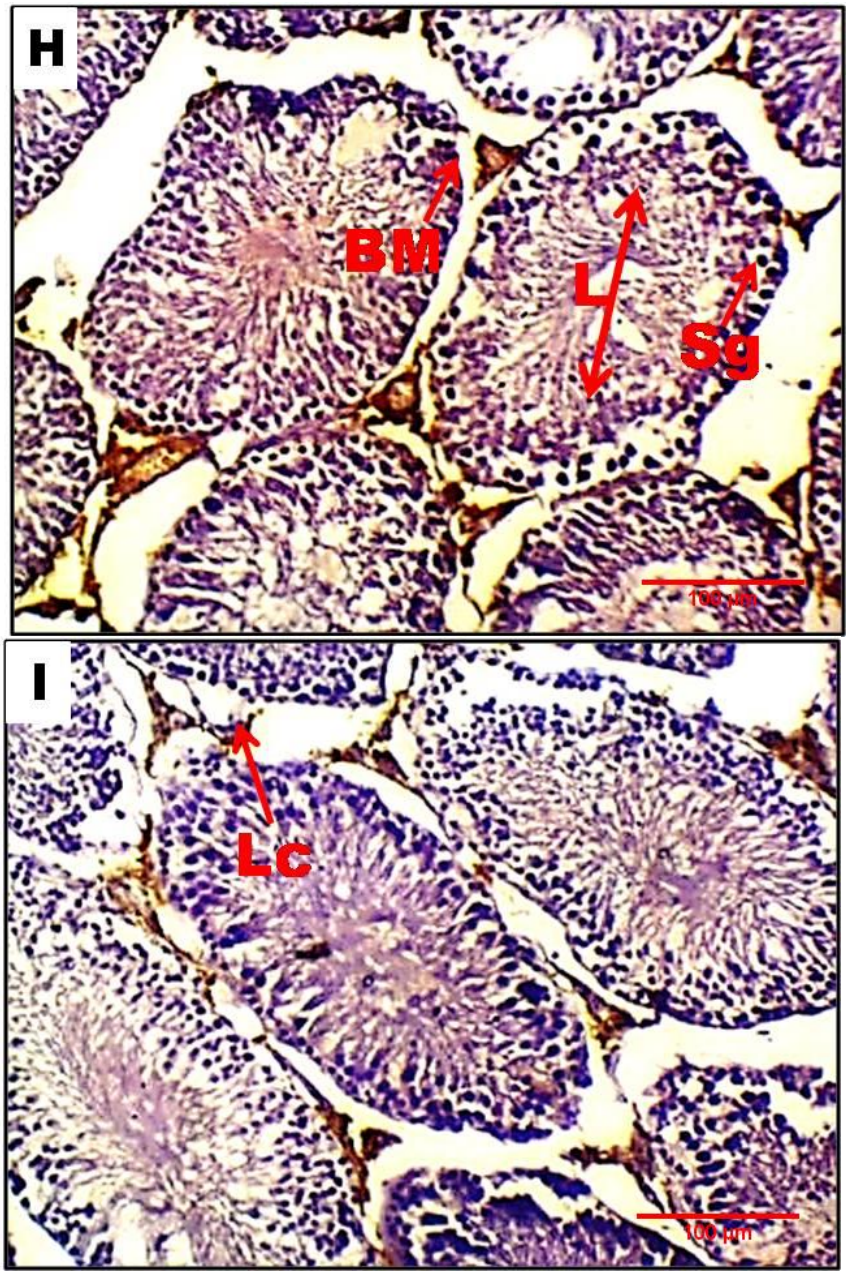
Immunohistochemical staining for Ki-67 in groups D and E (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the leydig cells (Lc) and sertoli cells (Sc).

Figure 4.7.1 depicts immunohistochemical staining for Ki-67 in groups D and E treated with RES (2.5 mg/kg and 5 mg/kg respectively). Moderate Ki-67 immunoreactivity, characterized by mild arrest of differentiation of spermatogenic cell lines was observed here.



Immunohistochemical staining for Ki-67 in groups F and G (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the leydig cells (Lc) and sertoli cells (Sc).

Figure 4.7.1 depicts immunohistochemical staining for Ki-67 in groups F and G treated with RV (150 mg/kg and 300 mg/kg respectively). Both groups F and G expressed strong positive Ki-67 immunoreactivity, which presented deeply stained dark brown granules characterized by normal proliferation and differentiation of spermatogenic cell lines.

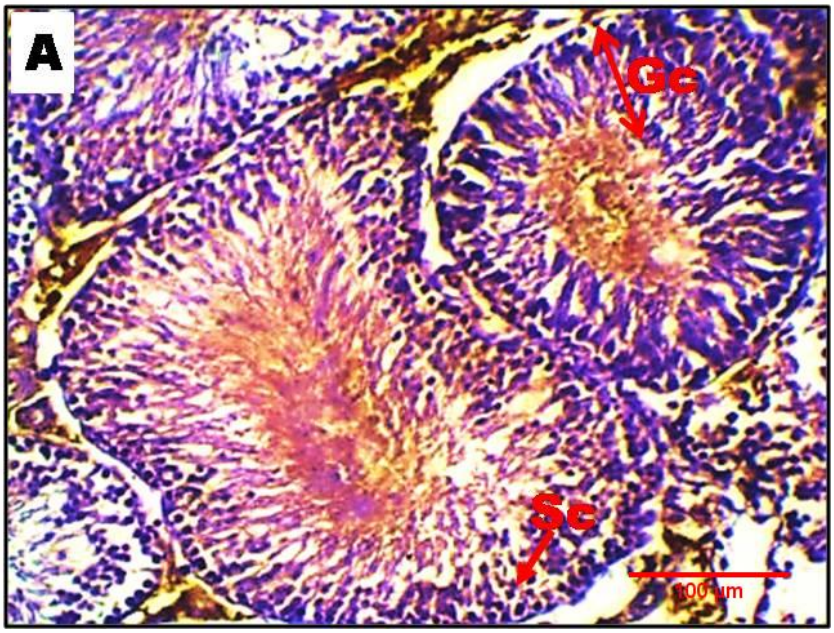


Immunohistochemical staining for Ki-67 in groups H and I (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the basement membranes (BM), lumen (L) leydig cells (Lc) and spermatogonia cells (Sg).

Figure 4.7.1 depicts immunohistochemical staining for Ki-67 in groups H and I treated RAZ (2.5:5:100 mg/kg and 5:10:200 mg/kg respectively). Only group H expressed strong positive Ki-67 immunoreactivity, which presented deeply stained dark brown granules characterized by normal proliferation and differentiation of spermatogenic cell lines while group I presented with

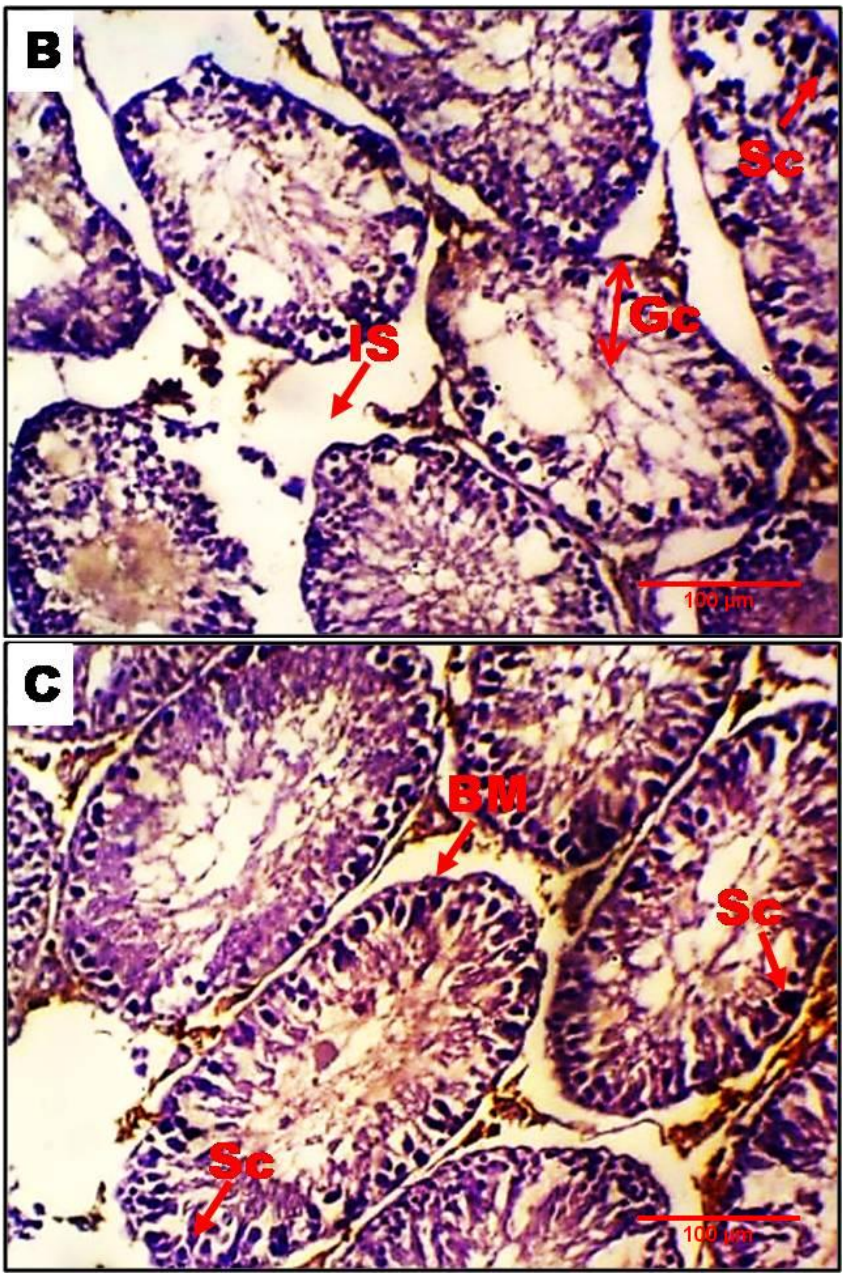
weak Ki-67 immunoreactivity, characterized by mild arrest of differentiation of spermatogenic cell lines.

4.7.2 Cytokeratin-18 Protein expression in Rats' Testes



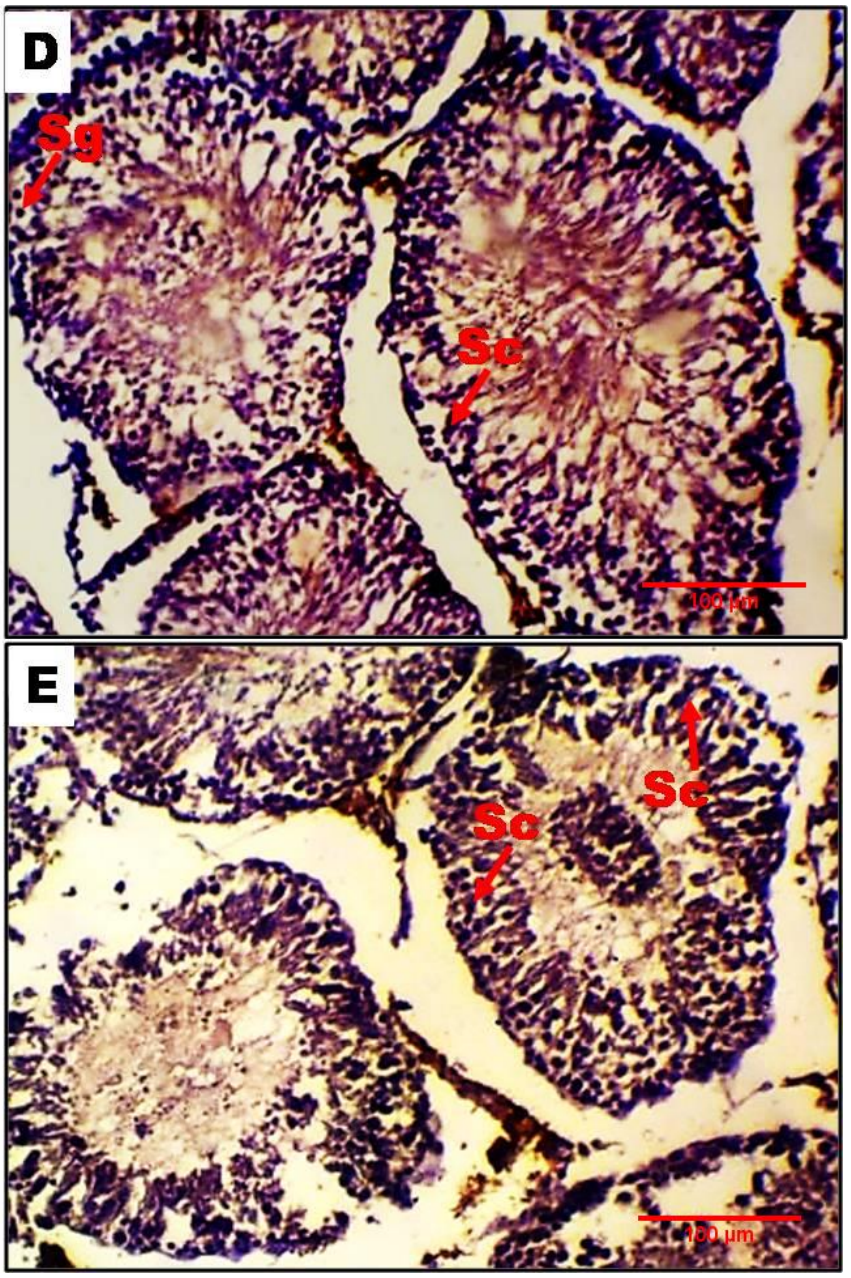
Immunohistochemical staining for cytokeratin-18 in control group A (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the germinal cells (Gc) and sertoli cells (Sc).

Figure 4.7.2 depicts immunohistochemical staining for cytokeratin-18 in control group A with negative immunoreactivity to cytokeratin-18 stain indicative of less immature sertoli cell index.



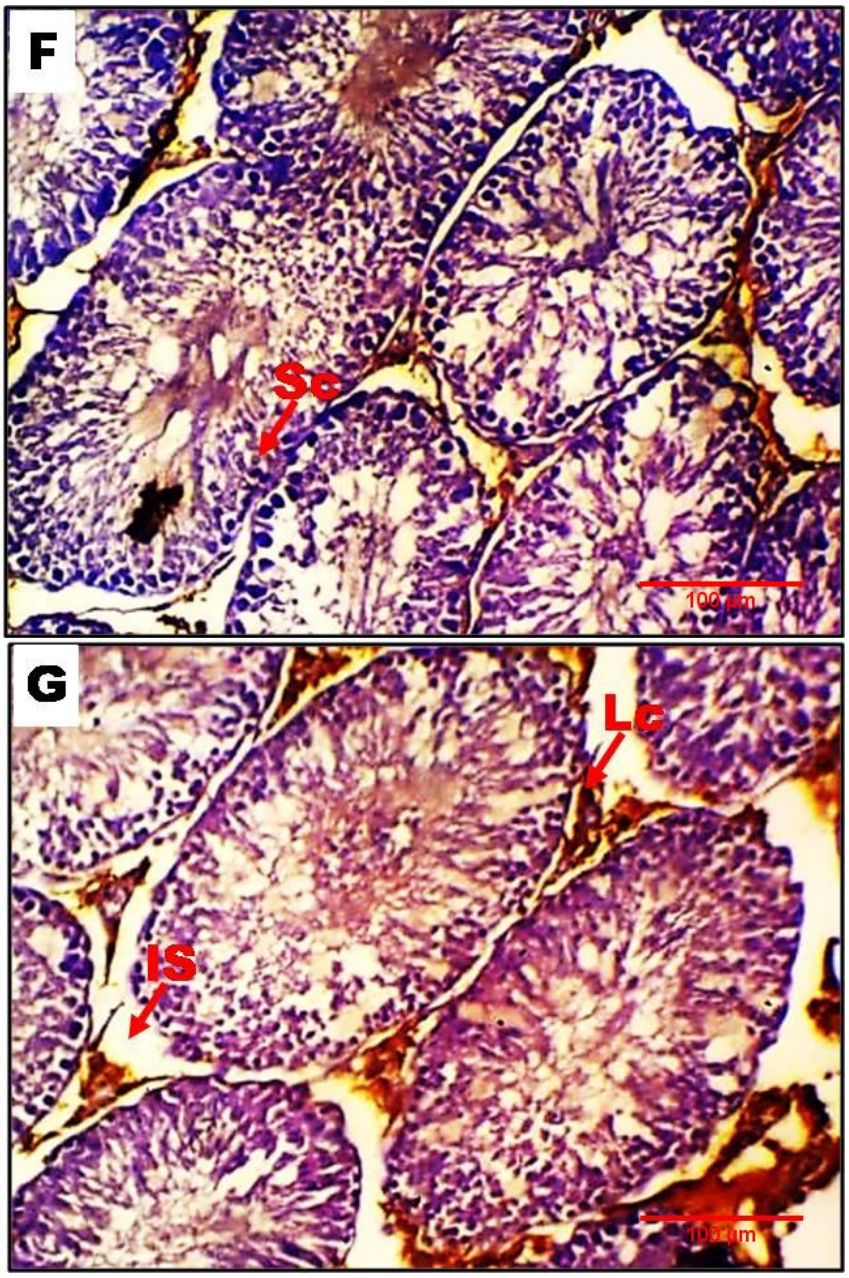
Immunohistochemical staining for cytokeratin-18 in groups B and C (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the basement membrane (BM), lumen (L), interstitial space (IS), germinal cells (Gc) and sertoli cells (Sc).

Figure 4.7.2 depicts immunohistochemical staining for cytokeratin-18 in groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively). The immunohistochemical staining appeared as deep dark-brown granules stained in the immature sertoli cells of groups B and C treated with CPZ which is a sign of prevalent immature sertoli cells localized at the periphery of the seminiferous tubules.



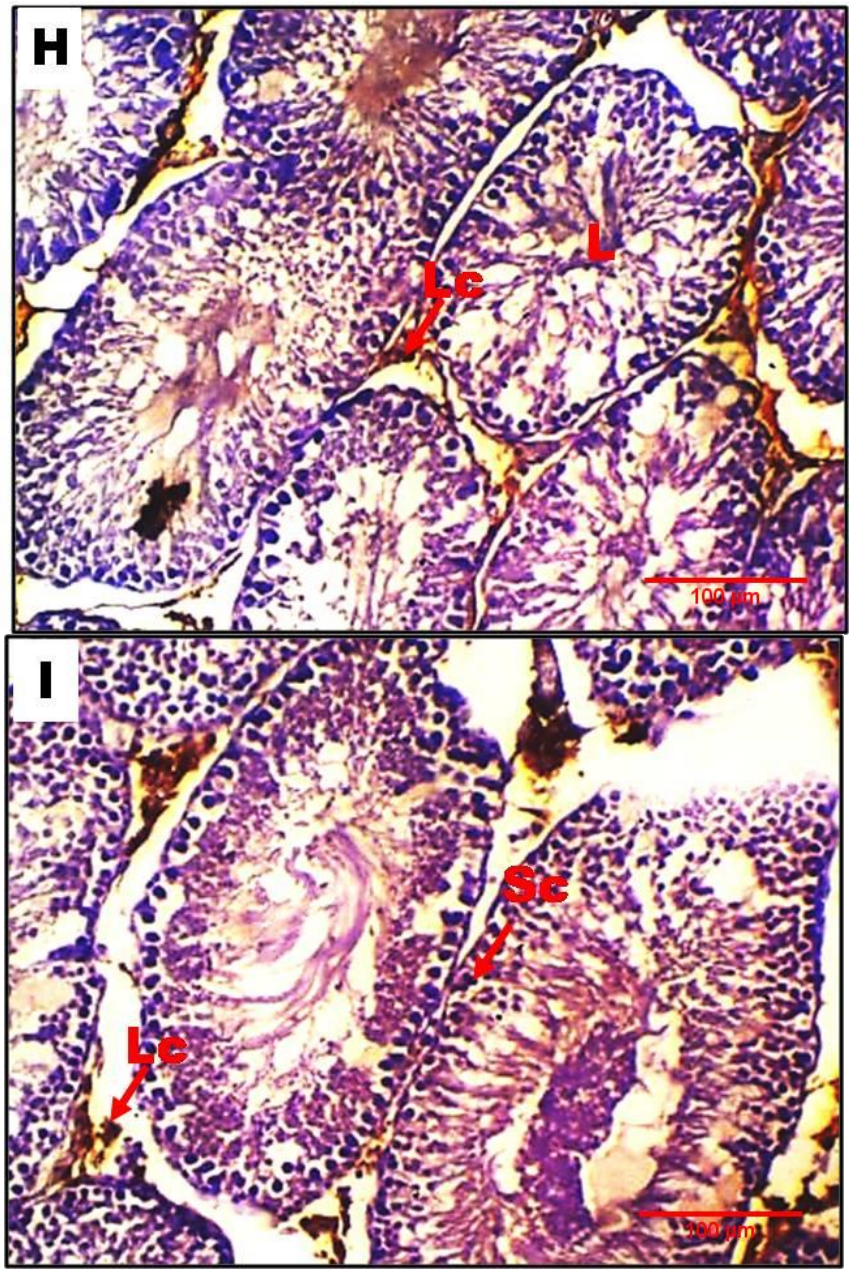
Immunohistochemical staining for cytokeratin-18 in groups D and E (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are spermatogonia cells (Sg) and sertoli cells (Sc).

Figure 4.7.2 depicts immunohistochemical staining for cytokeratin-18 in groups D and E treated with RES (2.5 mg/kg and 5 mg/kg respectively). The immunohistochemical staining appeared as deep dark-brown granules stained in the immature sertoli cells of groups D and E as well, indicating prevalent immature sertoli cells localized at the periphery of the seminiferous tubules.



Immunohistochemical staining for cytokeratin-18 in groups F and G (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the interstitial spaces (IS), leydig cells (Lc) and sertoli cells (Sc).

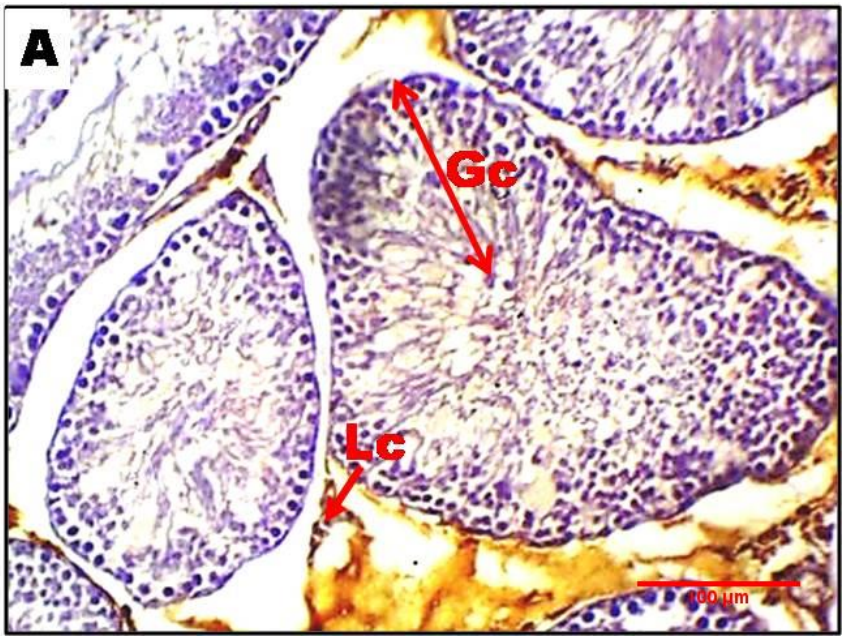
Figure 4.7.2 depicts immunohistochemical staining for cytokeratin-18 in groups F and G treated with RV (150 mg/kg and 300 mg/kg respectively). Group F expressed weak positive immunoreactivity to cytokeratin-18 stain with few immature sertoli cells localized at the periphery of the seminiferous tubules while group G expressed negative immunoreactivity to cytokeratin-18.



Immunohistochemical staining for cytokeratin-18 in groups H and I (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the lumens (L), leydig cells (Lc) and sertoli cells (Sc).

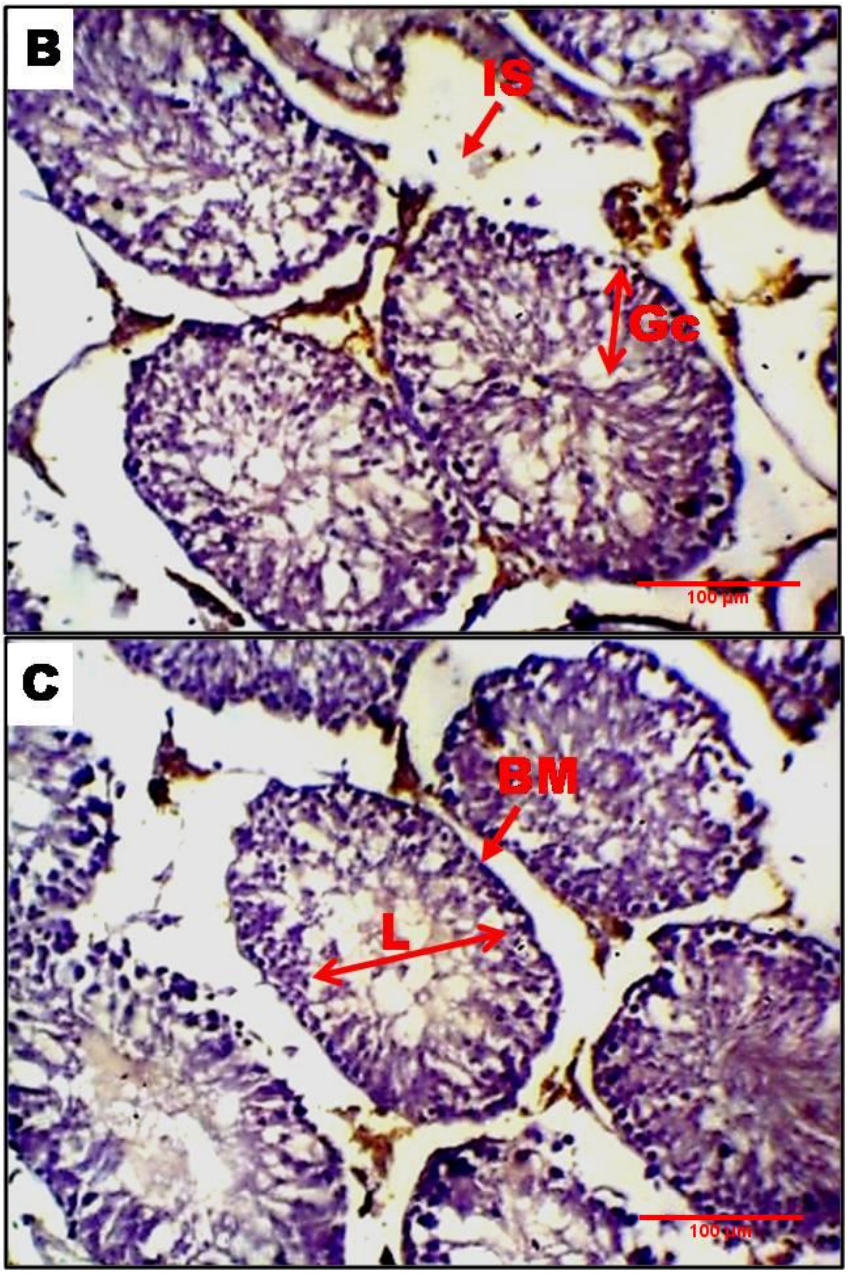
Figure 4.7.2 depicts immunohistochemical staining for cytokeratin-18 in groups H and I treated RAZ (2.5:5:100 mg/kg and 5:10:200 mg/kg respectively). Group H expressed weak positive immunoreactivity to cytokeratin-18 stain with few immature sertoli cells localized at the periphery of the seminiferous tubules while group I expressed negative immunoreactivity to cytokeratin-18.

4.7.3 Bcl_2 Protein expression in Rats' Testes



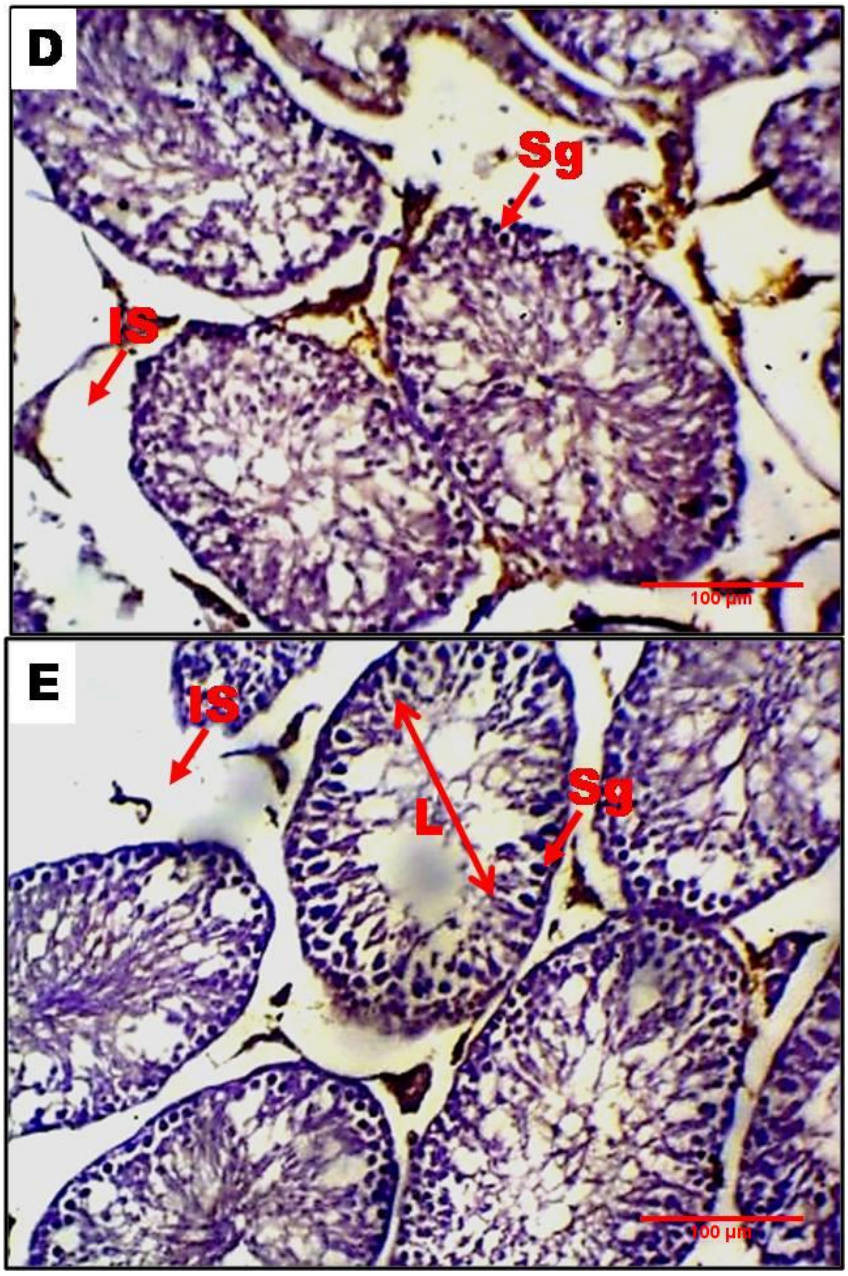
Immunohistochemical staining for Bcl_2 in control group A (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the germinal cells (Gc) and leydig cells (Lc).

Figure 4.7.3 depicts immunohistochemical staining for Bcl_2 in control group A with negative BCL-2 immunoreactivity which indicates reduced number of germinal cells death in the testes.



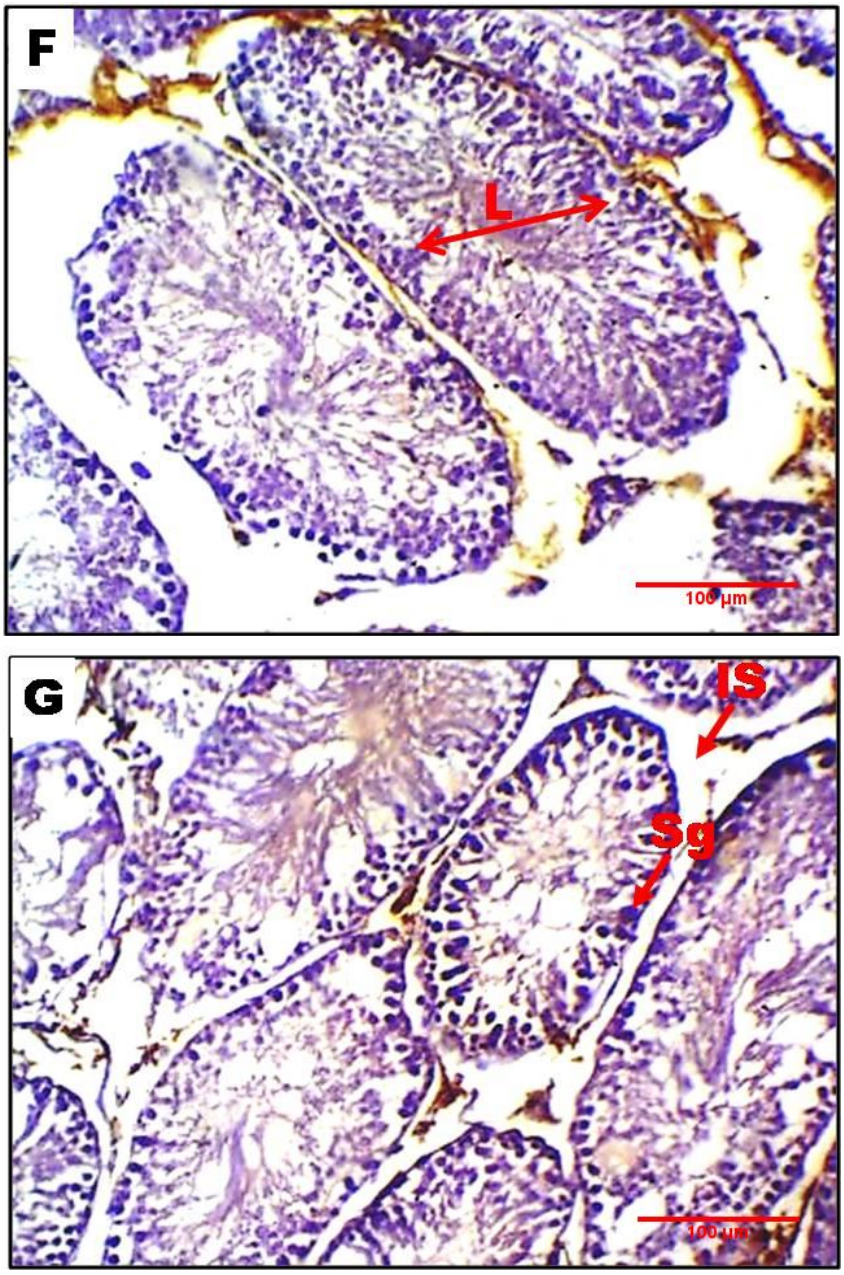
Immunohistochemical staining for Bcl_2 in groups B and C (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the germinal cells (Gc), basement memebrane (BM), and interstitial space (IS).

Figure 4.7.3 depicts immunohistochemical staining for Bcl_2 in groups B and C treated with CPZ (5 mg/kg and 10 mg/kg respectively). Both groups B and C showed strong positive immunoreactivity, presented with deeply stained dark brown germinal and leydig cells which is indicative of a high germinal cells apoptotic index in the testes.



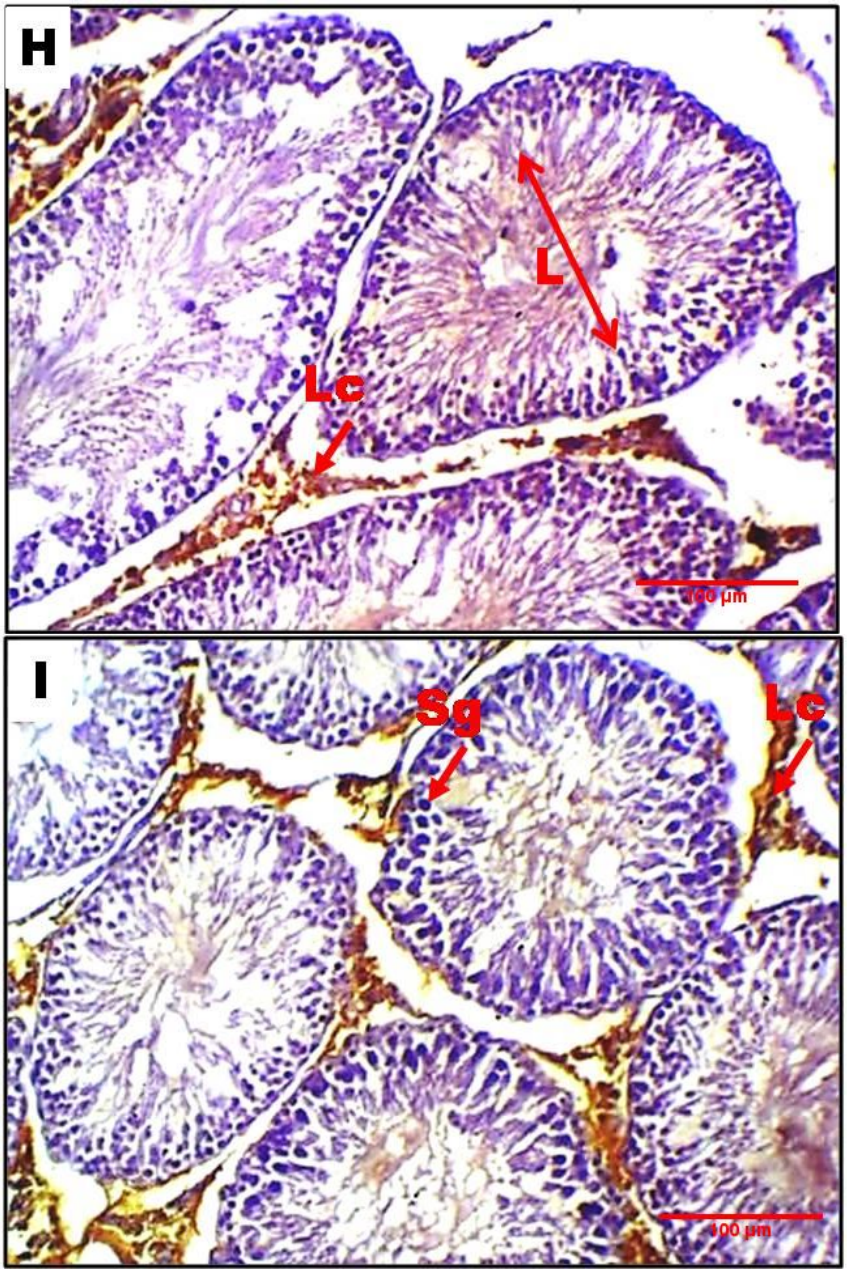
Immunohistochemical staining for Bcl_2 in groups D and E (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the spermatogonia cells (Sg), lumen (L), and interstitial space (IS).

Figure 4.7.3 depicts immunohistochemical staining for Bcl_2 in groups D and E treated with RES (2.5 mg/kg and 5 mg/kg respectively). Both groups D and E also presented with strong positive immunoreactivity has shown with deeply stained dark brown germinal and leydig cells which is indicative of a high germinal cells apoptotic index in the testes.



Immunohistochemical staining for Bcl_2 in groups F and G (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the spermatogonia cells (Sg), lumen (L), and interstitial space (IS).

Figure 4.7.3 depicts immunohistochemical staining for Bcl_2 in groups F and G treated with RV (150 mg/kg and 300 mg/kg respectively). Both groups F and G expressed a weak positive immunoreactivity, presented with lightly stained dark brown leydig cells to BCL-2 stain.



Immunohistochemical staining for Bcl_2 in groups H and I (Scale bar: 100 µm). Presented within and outside the seminiferous tubules are the spermatogonia cells (Sg), lumen (L), and interstitial space (IS).

Figure 4.7.3 depicts immunohistochemical staining for Bcl_2 in groups H and I treated RAZ (RAZ (2.5:5:100 mg/kg and 5:10:200 mg/kg respectively). Both groups H and I also expressed a weak positive immunoreactivity, presented with lightly stained dark brown leydig cells to BCL-2 stain.

4.8 GERMINAL CELL COUNT

4.8.1 Germ Cell Proliferation Index

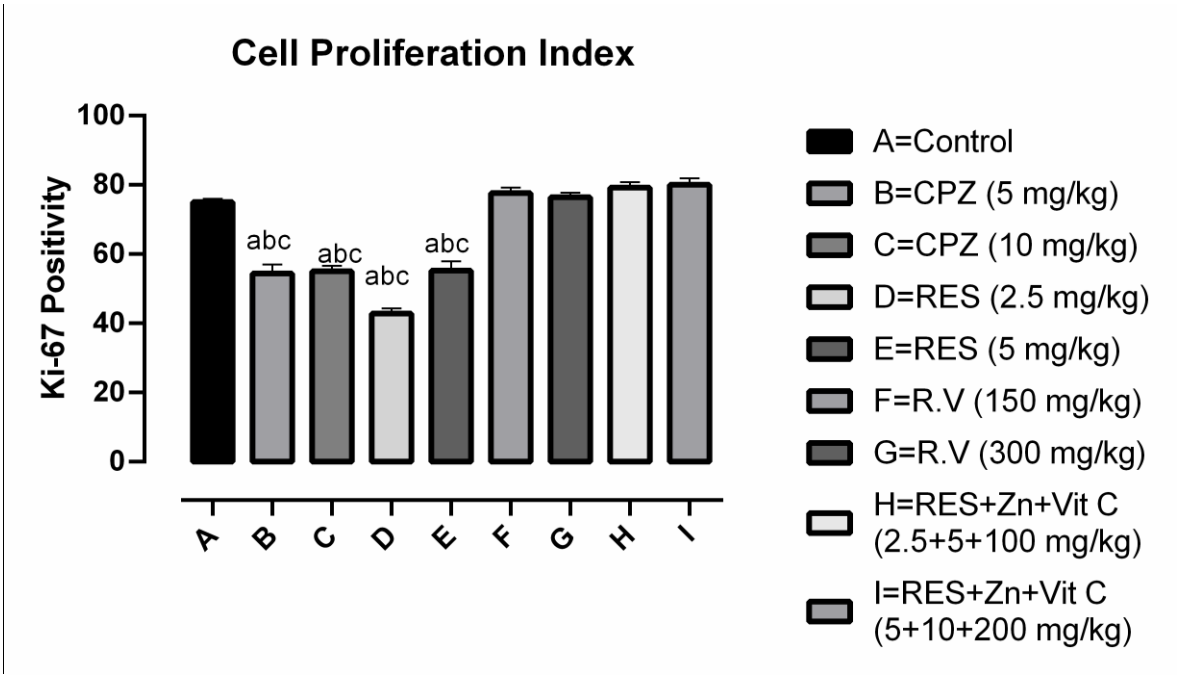


Figure 4.8.1: Showed comparison in germ cell proliferation index among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. *P<0.05 (n=5).

Figure 4.8.1 showed mean values of germ cell proliferation index among the groups after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. From the graph, significant decreased in germ cell proliferation index was observed when Chlorpromazine and Reserpine treated groups were compared with control group A.

Furthermore, slight significant increased in germ cell proliferation index was seen in *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups when compared with Chlorpromazine and Reserpine treated groups. No significant difference when compare Rauwolfia vomitoria and co-administration of Reserpine, Ascorbate and Zinc treated groups with the control group A.

4.8.2 Germ Cell Apoptotic Index

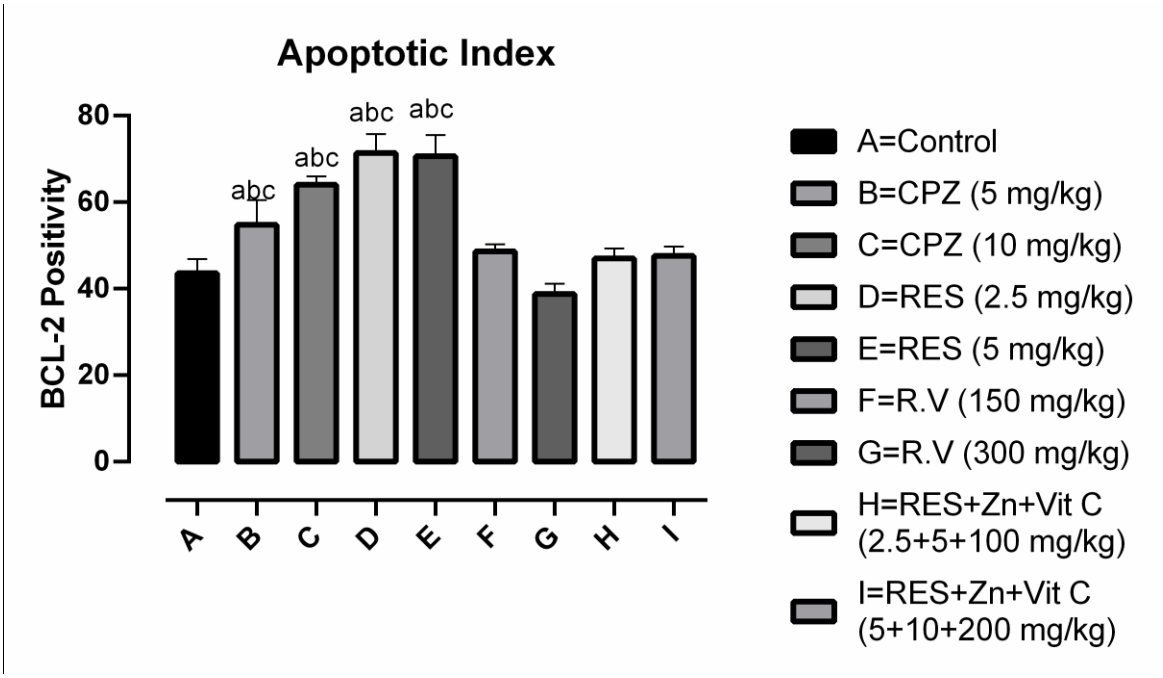


Figure 4.8.2: Showed comparison in germ cell apoptotic index among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. *P<0.05 (n=5).

Figure 4.8.2 showed mean values of germ cell apoptotic index among the groups after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. From the graph, significant increased in germ cell apoptotic index was observed when Chlorpromazine and Reserpine treated groups were compared with the control group A.

Furthermore, slight significant decreased in germ cell apoptotic index was seen in *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups when compared with Chlorpromazine and Reserpine treated groups. No significant difference when compare *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups with the control group A.

4.8.3 Sertoli Cell Index

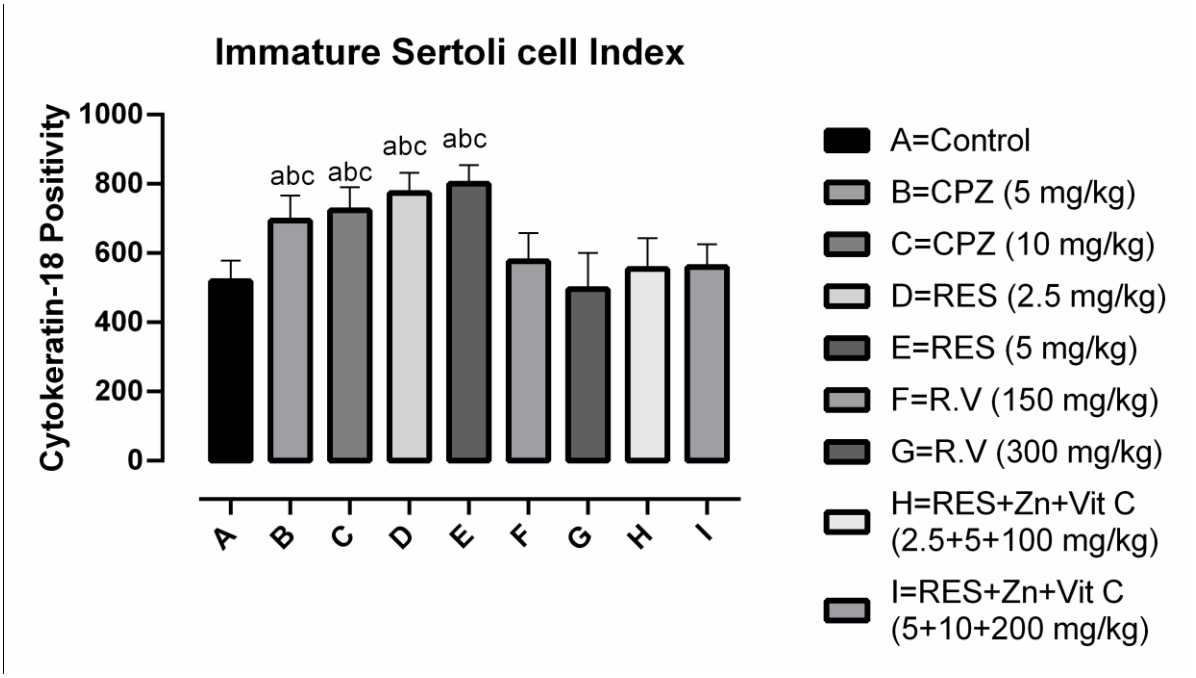


Figure 4.8.3: Showed comparison in immature sertolic cell index among the groups after the administration of CPZ, RES, *RV* and Co-administration of RAZ. **a** = Comparison with Control Group A; **b** = Comparison with Group H and **c** = Comparison with group I. *P<0.05 (n=5).

Figure 4.8.2 showed mean values of immature sertoli cell index among the groups after the administration of Chlorpromazine, Reserpine, *Rauwolfia vomitoria* and Co-administration of Reserpine, Ascorbate and Zinc. From the graph, significant increased in immature sertoli cell index was observed in Chlorpromazine and Reserpine treated groups when compared with the control group A.

Furthermore, slight significant decreased in immature sertoli cell index was seen in *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups when compared with Chlorpromazine and Reserpine treated groups. No significant difference when compare *Rauwolfia vomitoria* and co-administration of Reserpine, Ascorbate and Zinc treated groups with the control group A.

5.0 DISCUSSION

Infertility is a problem which has gained increased attention over the past several decades. While many factors may contribute to infertility, some medications may also affect reproductive functioning and thus may have an impact on fertility. Male infertility (abnormal spermatogenesis) has been more pressing issue than now, thus promoting an urgent need to solve this fertility deficit. Synthetic antipsychotic drugs like chlorpromazine and reserpine are one of the medications that have been reported to induce various side effects on the body system including reproductive system while psychiatric patients treated with traditionally used antipsychotic herbs like *Rauwolfia vomitoria* showed no traces of reproductive toxicity. This study highlights the effectiveness of some antipsychotic target compounds in driving the expression of some putative genes involved in normal spermatogenesis.

The morphometric data obtained from this study indicates significant increased in the body weight of animals's treated with Chlorpromazine and Reserpine while animals's treated with Rauwolfia vomitoria showed slight significant weight lost and co-administration of reserpine, zinc and ascorbate shows no significant different when compared with the control group. This result corroborate with the work of Raji *et al*., 2005 and Ajao *et al*., 2017 where they reported significant weight gained in the rats treated with chlorpromazine and also with Mokutima *et al*., 2009 and Dieudonne *et al*., 2014 that reported slight reduction in animals's body weight treated with Rauwolfia vomitoria. The weight gained by animals treated with chlorpromazine and reserpine might be as a result of blockage of the adrenergic alpha 1 receptor, M1 and M2 muscarinic acetylcholine receptors on gastric tissues thereby increases nutrient absorption and appetite stimulation (Raji *et al*., 2005). Moreover, slight weight lost in R.vomitoria might be as a result of the way R.vomitoria affects fat and carbohydrates metabolism. Reduction in the body weight might be due to the presence of alkaloids in the extract. Several lines indicated that

alkaloids could alter cells growth (Hiroshi *et al*., 2004) and lead to inhibit food intake. Therefore the amount of nutrients absorbed by animals treated with aqueous extract of R.vomitoria would not have been sufficient to properly cover their need for growth.

The cAMP responsive element modulator (CREM) is a transcription factor from the basic domain-leucine zipper family that binds to a regulatory palindromic DNA sequence called the cAMP response element in homo and heterodimers (CRE). CRE is found in the promoter regions of genes that respond to cAMP. In the absence of CREM-dependent transcription in post-meiotic germ cells, spermatid differentiation and apoptosis are inhibited (Hogeveen and Sassone-Coris, 2006). A cAMP responsive element (CRE) serves as a binding site for the transcription factor cAMP-responsive element modulator (CREM) in many spermatid-specific genes (Sassone-Corsi, 1995). Since male mice lacking a functional CREM gene are sterile due to round spermatid maturation arrest, CREM is necessary for spermatogenesis (Blendy *et al*., 1996; Nantel *et al*., 1996). Infertile men with round spermatid maturation arrest have a significant reduction or total loss of CREM protein (Weinbauer *et al*., 1998) and CREM mRNA (Steger, 1999). The CREM gene produces functionally different proteins with either activating or repressing capacity on target gene expression due to alternative transcriptional start sites, alternative transcript splicing, and alternative translational start sites (Behr and Weinbauer, 2001; Gellersen *et al*., 2002; Daniel *et al*., 2006). In a number of cases of idiopathic male infertility, Weinbauer *et al* reported that changes in CREM expression interfered with spermatid maturation (Weinbauer *et al*., 1998).

Protamines (post-meiotic nuclear proteins) are Arginine-rich nuclear proteins. Protamine replaces histones in the late haploid process of spermatogenesis, assisting in the stabilization of sperm DNA and sperm head condensation. During spermatogenesis, the chromatin structure and compactness of haploid spermatids undergo changes (Steger, 1999) whereas, the deoxynucleic acid (DNA)-histone bond in the round spermatid will be substituted with transition proteins,

while the transition protein in elongated spermatids will be substituted with protamine. Thus, changing from histone to protamine stimulate spermatozoa chromatin condensation (Steger *et al*., 2001).

Protamine has been shown in studies to be essential for male fertility. Insufficient PRM-1 and PRM-2 concentrations have been linked to infertility in women who are subfertile or severely infertile (Oliva, 2006).

CREM, PRM I, and II gene expression were examined in the groups after administration of Chlorpromazine, Reserpine, Rauwolfia vomitoria, and Reserpine, Zinc, and Ascorbate in combination. When compared to the control group, the Chlorpromazine and Reserpine treated groups displayed substantial downregulation of CREM and PRM II gene expression. This finding is consistent with Raji *et al*. and Khazan *et al*., who found that Chlorpromazine and Reserpine, respectively, had anti-fertility effects (Khazan *et al*., 1960; Raji *et al*., 2005). Moreover, slight significant up regulation of CREM, PRM I and II genes were observed in the groups F and G treated with traditionally used antipsychotic drug (*Rauwolfia vomitoria* leaves extract). Furthermore, CREM, PRM I, and II gene expression were significantly upregulated in groups H and I treated with a combination of Reserpine, Zinc, and Ascorbate. The upregulation of CREM gene expression in groups F, G, H, and I may be due to the presence of antioxidant compounds (Zinc and Ascorbate) in the drugs administered to these groups. This finding supports the findings of Sedigheh *et al*. and Sheida *et al*., who found that ascorbic acid is an effective antioxidant that helps prevent sperm defects and improves sperm motility (Sedigheh *et al*., 2016; Sheida *et al*., 2017). Oyewopo *et al*., 2010 also reported that zinc supplementation abate sperm parameters (sperm motility, count and morphology) toxicity induced by Propoxur in wistar rat model. Possible mechanism of protection conferred by zinc supplementation might be as a result of zinc metallothionein induction (Oyewopo *et al*., 2010).

CREM deficiency has been shown to affect protamine expression, resulting in infertility due to a disorder in round spermatid maturation (Blendy *et al*., 1999). Blendy *et al*., 1999 identified a connection between putative genes that enhance male normal fertility, and the findings of the current study's gene-regulatory dependent approach corroborate this.

As compared to control group A, serum FSH, LH, Testosterone, and hypothalamic GnRH concentrations were significantly reduced in high dose synthetic antipsychotic treated groups C and E (Chlorpromazine and Reserpine, respectively), although there was no substantial difference in Rauwolfia vomitoria treated groups F and G. Furthermore, in the co-administration of Reserpine, Zinc, and Ascorbate treated groups, there was a significant increased in serum FSH, LH, Testosterone, and hypothalamic GnRH concentration. Decreased in concentrations of serum FSH, LH, Testosterone, and hypothalamic GnRH observed in chlorpromazine and reserpine treated groups are consistent with those reported by Zamani *et al*, who found a significant increase in serum prolactin and a significant decrease in testosterone and Luteininzing Hormone after Chlorpromazine treatment in rats (Zamani *et al*., 2015). Furthermore, serum prolactin concentrations were significantly higher in the high-dose Chlorpromazine and Reserpine-treated groups (C and E), confirming Nira and Robert's findings that depletion of dopamine concentration hitting lactotroph cells results in hyperprolactemia, and hyperprolactemia often has a negative feedback on GnRH secretion in the hypothalamus (Nira *et al*., 2001).

Reproductive toxicity induced by Chlorpromazine and Reserpine from the results above might be as a result of dopaminergic fibers extending from the ventral tegmental area's A13 and A14 to the paraventricular and medial preoptic nuclei, respectively (Weiner and Molinoff, 1989). Antipsychotic drugs can alter pulsative output of GnRH hormones in the media preoptic region, with consequent effects on FSH and LH production in the adenohypophysis, by inhibiting or

depleting dopamine concentration in these hypothalamic areas. FSH has been identified as a key player in the transcription of the CREM gene, with the absence of CREM-dependent transcription in post-meiotic germ cells resulting in spermatid differentiation and apoptosis (Sassone-Corsi, 1998). CREM mRNA level is predominantly regulated by the FSH. FSH binds with G-protein alpha-s -coupled receptors, such as Follicle stimulating hormone receptor (FSHR) and activate Adenylate cyclase, Protein kinase A and cAMP-dependent PKA. CREM gene consists of CRE regions in the promoters and its expression is regulated by another cAMP responsive element binding protein 1 (CREB1); CREM expression is alternatively regulated by autoregulatory pathway (Monaco *et al*., 1995; Hogeveen and Sassone-Coris, 2006). CREM activity is control by a germ cell-specific transcriptional co-activator Four and a half LIM domains 5 (ACT) interactions. Kinesin family member 17 (KIF17), a germ cell-specific kinesin that controls ACT subcellular localization, regulates ACT's ability to control CREM behavior. In haploid spermatids, KIF17 colocalizes with ACT and mediates ACT transport from the nucleus to the cytoplasm at specific stages of spermatid maturation. KIF17 movement is modulated by phosphorylation by PKA (c-AMP-dependent). Neither the motor domain nor microtubules are necessary for KIF17 to shuttle between the nuclear and cytoplasmic compartments and transport ACT (Kotaja *et al*., 2005; Hogeveen and Sassone-Coris, 2006). As a result, CREM activation by ACT is responsible for the transcription of several important genes in postmeiotic germ cells, including PRM I and PRM II, which are both involved in DNA condensation and spermatid quality during spermatogenesis.

Male germ cells are more susceptible to oxidative stress than somatic cells for several reasons. One is their close proximity to the free radical-generating, phagocytic Sertoli cell (Beckman and Coniglio, 1979). Another is the inclusion of higher polyunsaturated fatty acids in their membranes. To protect them from oxidative injury, testicular cells are well-equipped with

enzymatic and low-molecular weight antioxidants to maintain redox homeostasis (Bauche *et al*., 1994). This defense includes superoxide dismutase (SOD) and glutathione peroxidase (GPX) activities, glutathione and antioxidant vitamins that can scavenge ROS or prevent ROS formation. Of these, SOD is considered as a first line of defense against oxygen toxicity and central regulator of ROS levels by catalyzing the dismutation of superoxide radicals to H2O2 and molecular oxygen. On the other hand, excessive ROS may over-ride antioxidant defenses or exceed scavenging ability of the antioxidant defense system leading to oxidative stress and permanent.

However, the activities of various intracellular antioxidant enzymes (SOD and GPx) that scavenge ROS and H2O2 to reduce oxidative stress were found to be significantly reduced while malondialdehyde concentration was significantly increased in chlorpromazine and reserpine treated groups. Earlier studies strengthened these findings of the present study which demonstrate the ability of chlorpromazine and reserpine to cause reduction in antioxidant concentration **(**Subakanmani *et al*., 2016**)** and increased in malondialdehyde concentration in animal's studies (Silvana *et al*., 2016). The decline in SOD activity is presumably due to strong interaction of chlorpromazine and reserpine with metal-binding moieties of SOD (Cu, Zn and Mn) **(**Casalino *et al*., 2002**).**

Furthermore, no significant differences were observed in Rauwolfia vomitoria and co-administartion of reserpine, ascorbate and zinc treated groups. Kujo, 2004 reported that Vitamins C and Zinc are well known antioxidants that can ameliorate oxidative stress-related testicular impairments in animal tissues (Ghosh *et al*., 2002; Thews *et al*., 2005; Marchlewicz *et al*., 2007). More also, Oyewopo *et al.,* 2010 reported that Zn administration minimized the evidences of testicular oxidative damage (GPx and MDA) induced by Propoxur in the rat. Supplementation of Zinc and ascorbic acid increases the availability of the vitamins to ameliorate oxidative stress.

This intervention could restore the activity of antioxidant enzymes to enable normal germ cell differentiation programs and protect spermatogenesis process. Furthermore, since chlorpromazine has been known to be effective antagonist of D2 dopamine receptors and similar receptors, such as D3 and D5 and reserpine to blocks the vesicular monoamine transporter (VMAT) (Henry *et al*.,1989) that normally transports free intracellular norepinephrine, serotonin, and dopamine in the presynaptic nerve terminal into presynaptic vesicles for subsequent release into the synaptic cleft. Blockage of dopamine receptors will make dopamine production of the neurons to drop substantially and dopamine will be removed from the synaptic cleft. From this study, dopamine concentration was significantly reduced in chlorpromazine and reserpine treated groups.

Spermatogenesis is a complicated process that involves mitotic, meiotic, and spermatogonial stem cell division and differentiation into mature spermatozoa. Mitosis proliferation of spermatogonial stem cells to produce spermatocytes, meiosis division of spermatocytes to form haploid round spermatids, and spermiogenesis conversion of round spermatids to mature elongated spermatids are all stages of the spermatogenesis process (He *et al*., 2009). From the sperm analysis, significant decreased in sperm parameters (sperm count, motile sperm and normal sperm morphology) were seen in chlorpromazine, reserpine and 150 mg/kg bw Rauwolfia vomitoria treated groups when compare with the control and co-administration of reserpine, ascorbate and zinc treated groups. These findings are in tandem with other researchers (Raji *et al*., 2005; Ajao *et al*., 2017). Correspondingly, Vit-C has been reported to augment sperm motility, guard spermatogenesis, and plays a vital role in semen integrity and fertility (Kini *et al*., 2009; Fernandes *et al*., 2011). The observed effects of Vit-C upon sperm motility are linked to both antioxidant and non-antioxidant, the cellular enzyme activity of the vitamin. Vit-C is essential to the production of sperm DNA content by being a coenzyme in DNA methylation.

The role of healthy DNA is a healthy spermatozoon (Nakada *et al*., 2006). It is worth noting that the motility of sperm depends much on mitochondrial quantities and qualities in the tailpiece. In particular, it was observed that Vit-C acts as a coenzyme to reduce the oxidative stress (Juan *et al*., 2004). This Vit-C behavior may, in one way or another, have led to the measurable result in sperm motility. Vit-C is an excellent electron source, giving an electron to free radicals such as superoxides and hydroxyls radicals, which reduces their reactivity (Levine *et al*., 2020).

Testicular cytoarchitecture has shown from this study revealed moderate to severe degeneration of seminiferous tubular epithelium in the rats treated with chlorpromazine and reserpine. More also, testicular immuno-staining of the chlorpromazine and reserpine treated groups showed positive immunoreactivity to Bcl_2, cytokeratin-18 staining intensity and weak positive immunoreactivity to ki-67 staining intensity. Testicular histoarchitecture were improved in Rauwolfia vomitoria and co-adminstration of reserpine, ascorbate and zinc treated groups. Previous studies demonstrated by Colager *et al*, 2009; Yamaguchi *et al*, 2009 and Deborah and Haim, 2020 support these findings. Low concentration of Zn in the diet, according to is a significant risk factor for low sperm quality and idiopathic male infertility (Colager *et al*., 2009). Moreover, Yamaguchi *et al*., said that Zn deficiency impedes spermatogenesis which is a major reason for sperm abnormalities (Yamaguchi *et al*, 2009). Zinc is necessary for making the outer membrane and tail of the sperm and also important for sperm maturation. Zinc supplements have been shown to improve sperm count, motility, form, function, quality and fertilizing capacity (Deborah and Haim, 2020).

In a similar way, hypothalamic neurons were severely degenerated with presence of red inflammatory cells (Fibrosis) and reduced cellular density in chlorpromazine and reserpine treated groups while Rauwolfia vomitoria and co-adminstration of reserpine, ascorbate and zinc treated groups showed numerous neurosecretory cells and increased cellular density. Several

investigators like Corniola *et al.*, 2008; Gower-Winter *et al*., 2013 have reported the mechanisms of neural proliferation under zinc supplementation and zinc-deficient conditions.

Stereological evaluations from this study revealed significant increase in germ cell apoptotic and immature sertoli index was observed in chlorpromazine and reserpine treated groups while germ cell proliferation index were significantly reduced. Rauwolifia vomitoria and co-administration of reserpine, ascorbate and zinc treated groups showed decrease in germ cell apoptotic and immature sertoli index and significant increase in germ cell proliferation. As reported by Croxford *et al*., 2011, Zn plays an important function in spermatozoa physiology. Primary testicular failure is caused by zinc deficiency, which reduces the activity of the luteinizing hormone receptor, reduces steroid production, and damages Leydig cells due to oxidative stress. (Croxford *et al.*, 2011).

Zn ion present in *Rauwolifia vomitoria* and co-administration of reserpine, ascorbate and zinc treated groups might able to mitigate reproductive toxicity effects of alkaloid (reserpine) by by binding to seminal plasma proteins and protecting sperm chromatin stability. This ion is involved in the formation of S-Zn-S type bonds in protamine structure, which helps to keep chromatin stable (Schneider *et al*., 2009). Zn is secreted in two forms: free and as part of protein complexes with a high molecular weight (Mogielnicka-Brzozowska and Kordan, 2011). Zn is abundant in the tails of mature spermatozoa, where it is linked to sulfhydryl groups and disulfide linkages (Schneider *et al*., 2009). By forming a specific number of SH-Zn-SH structures in the sperm nucleus, Zn controls disulfide cross-links (Khosronezhad *et al*., 2015). Zn is involved in the formation of coagulum, DNA stability control, antibacterial action and sperm movement inhibition, and .

5.1 CONCLUSION AND RECOMMENDATION

Healthy and quality spermatozoa are parts of prerequisite to ascertain fertility in men, and changes in the signaling pathways of the genes involved in spermatogenesis may result in spermatozoa of poor quality. This research has revealed the reproductive toxicity of synthetic antipsychotic drugs (Chlorpromazine and Reserpine), as well as the activity of compounds contained in the commonly used antipsychotic herb (Rauwolfia vomitoria) through crude extract administration and concurrent administration of isolated phytochemicals (Reserpine, Zinc, and Ascorbate) to reduce the danger of infertility always associated with antipsychotic drugs by upregulating the CREM, PRM I and II signaling pathways; increased sperm count, motility, normal sperm morphology, FSH, LH and testosterone hormonal concentration while antioxidant concentration (SOD, GPx) were also boosted. The study recommended that combination of RAZ should be prescribed in order to improve reproductive toxicity associated with antipsychotic drugs.

5.2 CONTRIBUTION TO THE BODY OF KNOWLEDGE

The present study has contributed to the body of knowledge in the following ways;

i. established that combination of RAZ confers best antipsychotic therapeutic effects on male reproductive parameters without the fear of coming down with infertility problems usually associated with antipsychotic drugs

ii. that pharmacodynamic synergy of combination of RAZ at lower dosage (2.5:100:5 mg/kg bwt) confers better therapeutic effects on male reproductive parameters when compared with higher dosage (5:200:10 mg/kg bwt). Though this effects are not statistically significant to each other.

iii. gives limelight to the pharmaceutical companies to developing new antipsychotic drugs that will be based on combining RAZ (reserpine {a potent antipsychotic drug}

with potent minerals that has been established to be beneficiary to reproductive system {ascorbate and zinc).

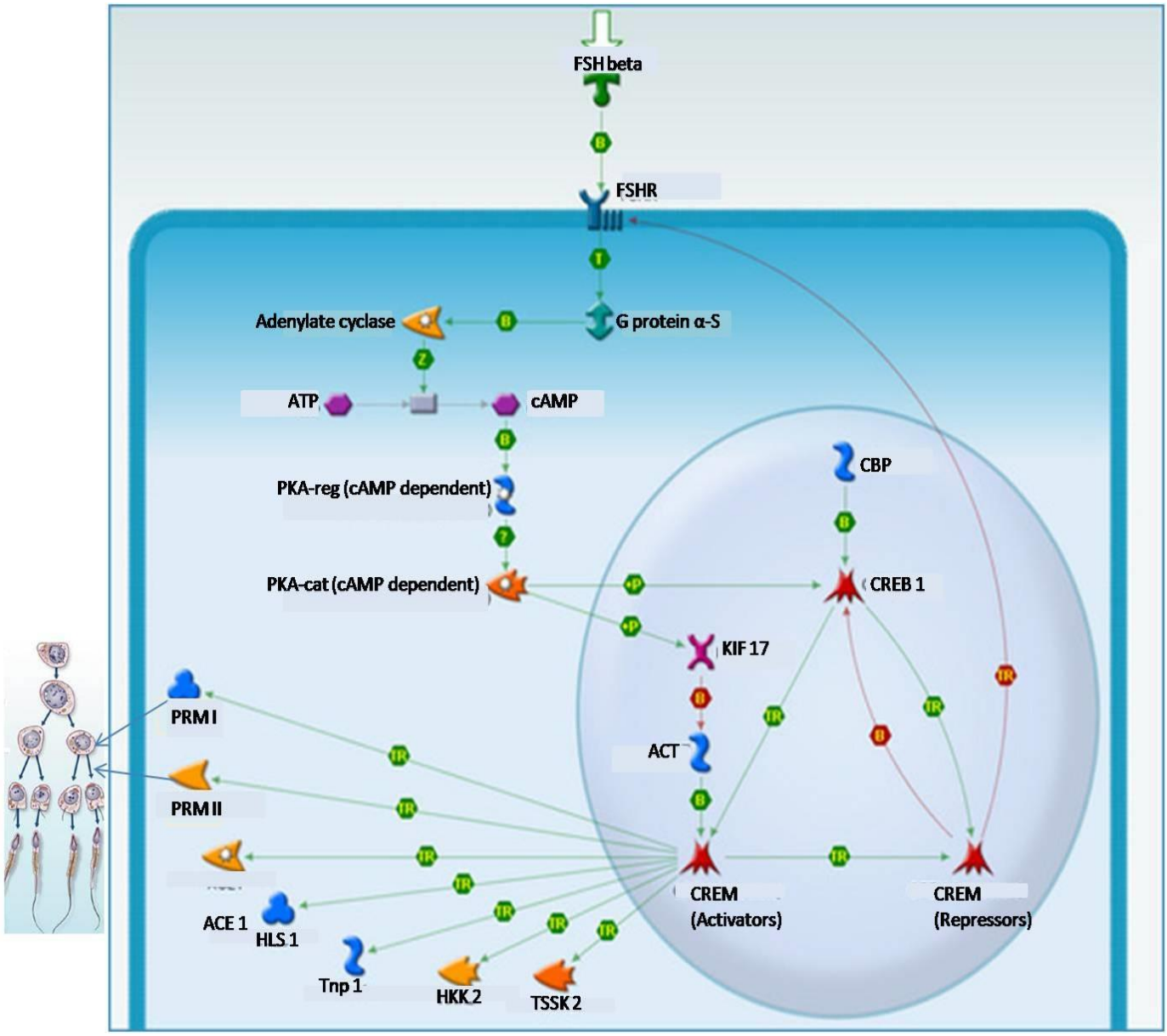


Figure 5.1 shown FSH, CREM, Protamine signaling pathways in ensuring normal spermatogenesis process (Hogeveen and Sassone-Coris 2006).

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