EFFECTS OF MATERNAL EXPOSURE TO *RICINUS COMMUNIS* OIL ON GENERATIONAL REPRODUCTIVE INDICES IN WISTAR RATS

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ABSTRACT

Increasing frequency of reproductive disorders has raised concerns on effects of endocrine disruptors on reproduction. *Ricinus Communis* oil (RCO) possesses laxative, contraceptive, labour inducing, cosmetics and estrogenic properties. There is paucity of information on generational reproductive effects of maternal exposure to RCO despite its estrogenic properties. This study was designed to investigate the generational reproductive effects of maternal exposure to RCO in rats.

Methanol extract of RCO was prepared by cold extraction and subjected to physicochemical analysis, gas chromatography and molecular spectroscopy. Acute oral toxicity was done by limit test procedure. Twenty five pregnant rats were randomly assigned to 5 groups; treated (p.o) with distilled water (control), RCO (950mg/kg) during Gestation Days (GD) 1-7, 7-14, 14-21 and 1-21 respectively. Maternal haematological, hormonal, biochemical, and histopathology of reproductive organs were assessed by conventional methods. Litter size, weight, morphometric data, Anogenital distance, pubertal age, sperm parameters, hormonal profile, organ weight and histopathology were determined in the F_1 and F_2 filial generations. Fertility tests, gestation, live birth, day survival and lactation indices were also determined by conventional methods. Data were analyzed using Student's t-test at $p = 0.05$.

No lethality was observed with RCO treatment up to 5000mg/kg. Physicochemical analysis of RCO gave values saponification (139.7) and density (0.95gm/ml) at 25^0C. Gas chromatography and molecular spectroscopy yielded ricinoliec acid (34.4%), methyl ricinoleate (46.7%), 9,12-octa-decadienoic acid (5.9%) and 9,17- octa-decadienal (12.9%). Serum alanine aminotransferase of GD 7-14 and 14-21 decreased significantly compared with control. Aspartate aminotransferase decreased significantly in GD1-7, 7-14, and 1-21. Total cholesterol, triglyceraldehyde and high-density lipoprotein increased while progesterone and oestrogen levels decreased significantly in RCO treated groups. The F_1 female from GD1-7 and male pups from GD1-7 and 7-14, showed significant decrease in birth-weight and morphometric data. Anogenital distance decreased significantly in RCO treated F_1 male (0.37±0.01, 0.39±0.02, 0.35±0.01, 0.39±0.01cm) compared with control (0.46±0.01cm). Pubertal age of F_1 female decreased significantly (42.2±1.2d) compared to control (49.0±2.3d). At postnatal day 90, F_1 males from RCO treated rats showed significant decrease in testis and body weight (0.98±0.03, 182.0±4.9g) compared with control (1.34±0.07, 234.0±5.1g). There were significant decreases in epididymal sperm count

(99.6±6.0, 88.4±5.6, 106.8±3.7, 79±6.3 X10^6ml) compared with control (125.4±6.9X10^6ml), and motility (70.0±4.5, 64.0±2.4, 66.0±5.1, 60.0±3.2%) compared with control (93.0±1.2). Abnormal spermatozoa in RCO treated groups increased significantly (12.8±0.2, 12.2±0.2, 13.6±3.2, 15.5±2.8%) compared with control (10.5±0.2%). Serum follicle stimulating and luteinizing hormones significantly increased in F_1 males of RCO treated groups. Estrogen levels significantly increased in F_1 males for days 7-14. Testosterone levels significantly decreased (27.1±6.7, 13.5±3.2, 15.4±2.8, 12.1±1.5nmol/ml) compared with control (54.3±3.8nmol/ml) in RCO treated F_1 males which also showed testicular interstitial oedema, reduced seminiferous tubular lumen and epididymal hypospermia. Mating, gestation, life birth, and day survival indices for control and GD 14-21 was 100% and 0% for others. Pairing F_1 males from RCO treated groups and untreated females yielded 100% for all indices. Only pubertal indices were altered in F_2 rats.

Maternal exposure to *Ricinus communis* oil at early gestation periods impaired estrogenic sensitive reproductive endpoints in first generation of rats.

Keywords: *Ricinus communis* oil, Estrogenic effects, Anogenital distance, Sperm parameters

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DEDICATION

“Say verily my solat (prayer), my sacrifice, my living and my dying are for Allah, the Lord of the Alamin (mankind, jinn and all that exist)”

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"He who does not thank Man will definitely fail in thanking the Creator (S.A.W.)"

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CERTIFICATION

I certify that **Salami, Shakiru Ademola** carried out this work titled**: Effects of maternal exposure to *Ricinus communis* oil on generational reproductive indices in Wistar rats** under my supervision in the department of Physiology, College of Medicine, University of Ibadan, Ibadan Nigeria.

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LIST OF ABBREVIATIONS

BW= Body weight

AGD= Anogenital distance

FSH= Follicle stimulating hormone

LH= Luteinizing hormone

RCO= *Ricinus communis* oil

AST= Aspartate aminotransferase

ALT= Alanine aminotransferase

VO= Vaginal opening

PPS= Preputial separation

GCMS= Gas chromatography and molecular spectroscopy

OECD= Organization of Economic Cooperation and development

F_1= First filial generation

F_2= Second filial generation

DNA= Deoxyribonucleic acid

GH= Growth hormone

IGF= Insulin like growth hormone

IUGR= Intrauterine growth restriction

HPA= Hypothalamic pituitary adrenal axis

GLC= Gas liquid chromatography

EPA= European protection Agency

S.C= Subcutaneous

P.O= per oral

EDC: Endocrine disrupting Chemicals

ISO= International Organization for Standardization

GHS= Globally Harmonized System of Classification and labeling of Chemicals

CHAPTER ONE

INTRODUCTION

David Baker pioneered the concept of fetal programming when from epidemiological data he was able to show an inverse relationship between birth weight and mortality due to adult ischaemic heart diseases (Baker, 1994a). Ever since then, the role of prenatal programming as determinant of adult diseases has become increasingly clear. Controlled animal based studies are beginning to provide insights into the molecular, cellular and systemic mechanisms that contribute to the several different manifestations of fetal programming.

The fetal programming hypothesis presupposes that a stimulus or insult acting during critical periods of growth and development may permanently alter tissue structure and function (Ozannes & Hales, 2002, Berthold, 2007). Programming is now known to be an important underlying feature of many systemic adult diseases including coronary heart disease, hypertension, insulin resistance syndromes and osteoporosis. While epidemiological evidence for the role of early developmental growth patterns leading to specific adult disease outcomes has continued to strengthen, experimental evidence from animal models has brought new and compelling support for this important determinant of lifetime health (Drake & Walker 2004). Zambrano *et al*., (2006) reported that maternal protein restriction resulted in delayed sexual maturation and premature ageing of reproductive function in offsprings**.** Savabieasfahani *et al*., (2006) also reported that prenatal exposures to Bisphenols have long-term differential effects on a variety of reproductive endocrine parameters that could impact fertility. While it is clear that fetal programming is not simply the effects of genes passed on through generations, it is clear that the phenotype of offspring subjected to a variety of intrauterine challenges is determined by lifelong gene expression patterns set into motion during critical windows of development and

that in some instances these phenotypes may pass on their effects to future generations (Langley-Evans *et al.* 1996; Barker, 1998; Nyirenda *et al.*, 1998; Doyle *et al.* 2000).

Nathaniesz, 1999 has identified ten essential principles of fetal programming namely:

1. There are critical periods of fetal development. Effects of physical, chemical, and biological influences will differ, often dramatically depending on timing.
2. Fetal programming produces longtime and typically permanent changes
3. Fetal programming produces structural changes in anatomy
4. Fetal programming is activity dependent.
5. The placenta often play a key role
6. While maternal, fetal and placental mechanism compensate for disturbances in the fetal environment, compensation produces secondary (typically negative) effects.
7. Postnatal effects of compensation may have further deleterious effects
8. Effects of the environment on the fetus are often different from those on adults or even infants.
9. Effects can be passed on to future generations
10. Effects frequently are different in males and females.

Increased occurrence of reproductive disorders has raised concerns regarding the impact of endocrine disrupting chemicals on reproductive health especially when such is during fetal life (Savabieasfahani *et al*, 2006). Societal concern has continued to mount over the potential deleterious effects on animal/human health of exposure to endocrine-disrupting compounds (EDCs). Endocrine disrupting chemicals are hormonally active, synthetic, or natural compounds that are present within our environment and food sources at concentrations that can interfere with the normal activity of endocrine systems/tissues, most notably the reproductive endocrine axis

(Crisp *et al*., 1998, IPCS, 2002). The controversy regarding the deleterious effects of EDC exposure has mainly been fueled by studies that point to likely effects on human health, including the recent dramatic increases in the incidence of estrogen sensitive cancers (breast, prostate and testicular) (Kelsey & Berstein 1966, Bergstrom *et al*., 1996), the decline in human sperm quality and quantity (Toppari *et al*.,1996), a notable rise in endometriosis (Crisp *et al*.,1998), an increase in genital abnormality in boys (Paulozzi *et al*., 1997), and early puberty (Herman-Giddens *et al*., 2001) in girls.

Endocrine disrupting chemicals that can interact with estrogen receptors have received considerable attention because they can modulate signaling by native estrogen, a key regulator of several physiologic functions including reproduction (Danzo, 1997). It has also been well established in human medicine that fetal exposure to the synthetic estrogen, diethylstilbesterol, has resulted in a wide variety of problems in the daughters of mothers prescribed diethylstilbesterol during pregnancy, including increased risk of cancers and infertility (Senekjian *et al*., 1988, Newbold, 2004).

Extracts from plants have been found to contain a multitude of biologically active compounds which have been ascribed various effects on health (Gustafsson, 2008). Phytoestrogen and Phytosterols are molecules with estrogenic and steroid activity that are found in plants, and because they have structural similarities with estrogen and steroids they have been found to interact with estrogen receptors in human cells (Anthony, 2003). Although these factors act through different mechanisms, their interactions with nuclear receptors have attracted a great deal of interest. In particular, those compounds which bind to estrogen receptors, the so called phytoestrogens, have been subject of much discussion in terms of their potential beneficial or possibly harmful effects on human health (Gustafsson, 2008). Phytoestrogen are weaker in

activity than steroidal estrogens. Estrogens act through two receptor isoforms, estrogen receptor α and estrogen receptor β, with often opposite effects. Interestingly, phytoestrogens in general have been reported to bind better to estrogen receptor β, a relatively recently discovered receptor whose important physiological functions are becoming better known (Gustafsson, 2008).

Soya (*Glycine max*) contains β sitosterol, daidzen and genistein which are isoflavones with estrogenic properties. Geometrical structure of daidzen compares with 12 β- estradiol and can therefore mimic its actions. *Pueraria mirifica* root a Thai plant used as a bust developing preparation when taken internally contain daidzen and genistein with other fascinating steroid like materials (Anthony, 2003). Avocado oil (*Persea gratissima*) contains phytosterols like β sitosterol, campsterols, stigmasterols, brasicasterols, tocopherols and other unidentified sterols. These naturally occurring sterols bear tremendous similarity to synthetic steroids like corticosterone and hydrocorticosterone. Methanolic extract of *Phaseolus vulgaris* which play an important role in Greek diet has been reported to exert a strong estrogenic effect (Tsiapara *et al*., 2008).

Ricinus communis oil is derived from the seed of the *Ricinus communis* plant. From the study of literature, *Ricinus communis* oil has found wide usage in cosmetics (over 700 different brands of cosmetic products (FDA, 2002). Concentration data obtained from an Industry survey by the Cosmetic, Toiletry, and Fragrance Association (CTFA 2004) indicated that *Ricinus communis* (Castor) Seed Oil is being used in cosmetics at concentrations of up to 81%. Food and Drug Administration (FDA, 2003a) has classified *Ricinus communis* as a stimulant laxative, *Ricinus communis* oil is thus available as "on the counter drug" (OTC). Most contraceptive jellies have also been found to contain ricinoliec acid which is the main component of *Ricinus communis* oil (Duke and Wain, 1981). Clinically, the use of *Ricinus communis* oil as a labour inducer has been

extensively reported (Davis, 1984, Mitri *et al*., 1987, Steingrub *et al*., 1988, Garry *et al*., 2000, Boel *et al*., 2010). The oil was also reported to have abortifacient activity when taken orally by pregnant women. Extracts of the seed have been tested in women and found to produce long-term contraception (Okwuasaba *et al*., 1991). Okwuasaba *et al*., (1991) also evaluated anticonceptive and estrogenic effects of a methanol extract of *Ricinus communis* var.*minor* seeds oil in rabbits and rats. In the study to determine estrogenic activity, endpoints used to estimate the estrogenicity of the Castor bean extract were uterine weight ratio, degree of vaginal cornification, and quantal vaginal opening. *Ricinus communis* oil was found to increase all these endpoints even in ovariectomized rats (Okwuasaba *et al*., 1991).

From the foregoing it is quite clear that *Ricinus communis* oil has been shown to possess laxative, contraceptive, labour inducing and estrogenic properties. Studies have also linked agents with estrogenic properties to having endocrine disrupting capabilities with resultant harmful effects on reproductive endpoints particularly when exposure is during fetal development (Bergstrom *et al*., 1996, Kavlock, 1998, 1999, Leonida *et al*., 2007).

Despite the reported extensive human use and estrogenic properties of *Ricinus communis* oil, its generational reproductive effects sequel to maternal exposure on reproductive functions in litter delivered has not been explored. The present study was therefore designed to investigate the effects of maternal exposure to *Ricinus communis* oil on reproductive functions in first and second filial generation of litter taking into consideration the concept of fetal programming.

Statement of the Problem:

Ricinus communis seed oil has been shown to possess laxative, contraceptive, labour inducing and estrogenic properties. Studies have also linked agents with estrogenic properties to having endocrine disrupting capabilities.

1. Could *Ricinus communis* oil due to its extensive human use and reported estrogenic properties serve as an endocrine disruptor after maternal exposure?
2. What is/are the critical period during gestation when maternal *Ricinus communis* oil exposure can be deleterious?
3. What are the likely effects on reproductive functions and organs in first (F_1) and second filial (F_2) generations as a result of maternal exposure to *Ricinus communis* oil?

Justification for the Study

1. Paucity of studies on generational reproductive effects of maternal exposure to *Ricinus communis* seed oil despite extensive use of *Ricinus communis* seed oil as laxative, cosmetics, anti-conceptive and labour inducer agent.
2. Need to adequately explore possible toxicological reproductive effects of maternal exposure to *Ricinus communis* seed oil in offspring of exposed mothers.

Aims and objective of study:

This study was therefore designed to:

1 Determine the effects of maternal exposure to *Ricinus communis* (RC) oil on maternal biochemical, hormonal, heamatological parameters and histopathology of reproductive organs.

2 Explore the generational reproductive effects of maternal exposure to RC oil

3 Determine whether maternal administration of RC oil will have effects on morphometric data (abdominal diameter, head diameter, body length), anogenital distance and index, size, weight, age of onset of puberty of litter.

4 Determine if maternal administration of RC seed oil will have effects on sperm parameters and fertility index later at puberty in offspring/litter of exposed mothers

5 Determine if maternal exposure to RC seed oil can have effects on reproductive hormones in offspring of exposed mothers

6 Determine the effects of maternal administration of RC seed oil on gestational length of exposed female rats among others

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Physiology of pregnancy

Pregnancy is said to occur when a healthy sperm fertilizes the ovum and then gets implanted. In mice, rats and various other species, sterile coitus, stimulation of the cervix with a glass rod or suckling a foster litter is known to initiate prolonged secretion of prolactin and retention of corpus luteum, with a consequent delay in the return of normal cycle for some time (Encyclopedia Britannica, 2009). This is called pseudo pregnancy. The period between the establishment and termination of pregnancy is called gestation period, and it is about 21 days in rats. From the moment of fertilization, all mechanism involved in the establishment, maintenance and termination of pregnancy are controlled by interplay of hormones produced in the mother and fetus (Encyclopedia Britannica, 2009).

2.2 Fertilization and early pregnancy

Fertilization of the ovum by the sperm usually takes place in the upper part of the fallopian tube. A number of factors have been implicated as speeding up fertilization by aiding sperm transport. For example during ovulation or estrous phase of a cycle, mucus is secreted by the cervix in greater amounts and it is less viscous with anisotropic properties which ensures that sperm entering the mucus move in a unidirectional manner along the tract (Tampion and Gibbon, 1962; Odebaid, 1968). Other substances like prostaglandin (Euler Von, 1965, Von Euler and Eliason, 1967) and oxytocin have been shown to aid sperm transport (Fox and Knaggs, 1969). On locating the egg, sperms stick to it by an agglutinating mechanism and fusion is mediated by fertilin a protein on the surface of the sperm head (Allen and Green, 1997).

The fusion of the egg and sperm also sets off a reduction in the membrane potential of the ovum that prevents polyspermy. During fertilization, the nuclei of the male and of the female germ cell meet and add their chromosomes. After fertilization, the cumulus is shed and the zygote begins to divide first into two, then to four, eight, sixteen, thirty- two until a mass of cell called morula is obtained. Later, a cavity develops in the morula and the resulting structure is called blastocyst (Roberts, 1980). The pattern of cleavage or division differs from one species to another depending on the amount of yolk in the zygote (Bannister *et al*, 1999). The time spent for the transport of blastocyst from the oviduct to the uterus varies between species but ranges between 2-5 days it remains unattached for about 3 days before implantation occurs (Sauer, 1979).

2.3 IMPLANTATION

This is the process by which the blastocyst becomes embedded in the lining of the uterus and establishes connection with the maternal circulation. Sauer, (1979) defined the start of implantation as the period at which it is possible to distinguish differences between those areas of the uterus, which do not contain a blastocyst. By the process of implantation, pregnancy is established; and it takes place on day 5-6 in small rodents, the evening of day 6 in rats (Psychoyos, 1973); day 13 in pigs (Crombie, 1970); day 18-22 in cow (Leiser, 1975) days 15 in sheep (Boshier, 1970) and days 5-7 in man.

The process of implantation requires that the egg reaches the precise stage of maturity (the blastocyst stage) and need to arrive the uterus at the secretory phase i.e. the hormone dependent changes resulting in the development of short-live receptive endometrium must have occurred. Psychoyos, (1973), Weitlauf, (1994) identified three separate loci reactions at the time of implantation. Firstly, there is an increase permeability of blood vessels close to the implantation

sites such that the site is stained blue after injection of macromolecular dyes like Evans blue, Pontamine sky blue or Greigy blue. This dye reaction is especially marked in rodents such as rats and mouse (Finn & McLaren, 1967) in which definitive implantation occurs but has also been used to assess initial stages of attachment in the sheep (Boshier, 1970). This reaction precedes any other macroscopic evidence of implantation in the uterus (Psychoyos, 1973, Evans and Kennedy, 1978). Since this response only occurs naturally at the time of maximum sensitivity to decidual induction and since the permeability reaction is thought to be an immediate pre-requisite to the decidual cells reaction and implantation (Yochim, 1975), it has been used to assess deciduogenic stimuli and the timing of implantation initiation.

The second reaction was described by Wilson, (1963) who demonstrated the passage of cells he called "primary invasive cells" from the blastocyst into the uterine epithelia and the third reaction is the appearance of the enzyme alkaline phosphatase in the endometrial stroma around an implanting blastocyst. Kennedy, (1997) has also reported experimental evidences for local interaction between blastocyst and endometrium at the time of implantation. The trophoblast of the blastocyst exerts an enzymatic, destructive influence on the swollen uterine lining, leading to erosion of both the superficial epithelium of the uterine lining and also its deeper, cellular connective tissue. This early stage of invasion ends in a few days; the blastocyst is then completely buried within a more superficial and compact layer of the total uterine lining. While the blastocyst is completing this phase of implantation, its original shell of cellular trophoblast steadily proliferates giving a multitude of cells that lose their outermost membranes and merge. The result is a thick peripheral layer consisting of a common mass of cytoplasm in which many nuclei are embedded. This external investment is called syncytial trophoblast. (Encyclopedia Britannica, 2009)

The mechanism of implantation has been studied in detail in rats and mouse and the subject has been reviewed. In all mammals studied, progesterone is essential for the maintenance of early pregnancy and in most cases it is also a pre-requisite for implantation. Progesterone has been shown to be essential in initiation of embryo implantation and maintenance of pregnancy in a number of mammalian species (Ghosh *et al*, 1997). Administration of anti-progestin (mifeprestone) was found to inhibit blastocyst implantation and pregnancy establishment (Nayak *et al*, 1998). Although it is established that both post-ovulatory ovarian estrogen and progesterone are pre-requisites for implantation in rat and mouse (Psychoyos, 1973) and that pre-ovulatory estrogen probably plays a role in potentiating their influence, a requirement for post-ovulatory estrogen in the implantation or attachment process in other species has not been established.

Estrogen triggers the uterus to produce growth factors such as epidermal growth factor (EGF), Heparin-binding –EGF (HB-EGF) and leukemia inhibitory factor. These proteins, in the presence of Hoxa-10 allow the expression of cycloxygenase (COX) enzymes (Paria *et al*., 2000). It was discovered in mice that these enzymes are critical in implantation (Lim *et al*, 1997; Paria *et al*, 2000). Since the uterus in mice defective of them cannot receive embryo (Lim *et al*, 1997; Paria *et al*, 2000), HB-EGF is also very important, as it is able to stimulate trophoblast growth and zonal hatching of the blastocyst (Martin *et al*., 1998).

2.4 Maintenance of pregnancy

A number of hormones produced by the endocrine system play vital roles in initiation and maintenance of pregnancy. In all viviparous animals progesterone is necessary for the sequence of events leading to conception. In some of these animals, there is a complete dependence on

corpus luteum, for the maintenance of pregnancy as it is the source of progesterone throughout. In other species however, the placenta takes over this function after its formation. The main way through pregnancy until parturition is not fully understood, as different schools of thought exist concerning this subject. However, it is known that there is a change of endocrine nature from a pre-implantation endocrine system, which is controlled by the maternal central nervous system, to a post-implantation endocrine integration. It is this post-implantation endocrine system that is necessary for maintenance of pregnancy, accomplishment of parturition, and the nursing of the newborn. Progesterone maintains pregnancy in rabbit through its inhibitory action on the myometrium (Rhoades and Bell, 2009)

The corpus luteum continues to function during pregnancy, supplemented (in eutherian, or placental, mammals but not in marsupials) by endocrine secretions of the placenta (the organ through which contact between mother and fetus is maintained). The hormonal activity of the placenta varies with the species; in man, for example, the placenta secretes two gonadotropins called human chorionic gonadotropin (HCG) and human placental lactogen (HPL). HCG, like the pituitary gonadotropins, is a glycoprotein, with a molecular weight of 25,000 to 30,000. HPL is a protein, with a molecular weight variously estimated at about 19,000 or 30,000. One or perhaps both of these hormones, which become detectable during the early weeks of human pregnancy, probably stimulate luteal secretion. After two months the human placenta begins to manufacture estrogen and progestin; as a consequence, the corpus luteum is no longer needed for the maintenance of pregnancy. Much of the estrogen, although synthesized in the placenta, is derived from a compound (dehydroepiandrosterone) formed in the adrenal glands of the fetus. The placenta and the fetus thus form an integrated endocrine complex, a striking index of the high level of specialization found in the regulation of mammalian reproduction.

The placenta probably secretes a luteotropin in all mammalian species, thereby contributing to prolongation of the life of the corpus luteum. In the mare and the monkey the placenta also secretes estrogen and progesterone, as in man, but in the mouse and rabbit it secretes only estrogen, and in the hamster and rat it secretes neither. In these last four species and in others like them, in which the placenta cannot substitute completely for the corpus luteum, ovariectomy (removal of the ovaries) of a pregnant female leads to the termination of pregnancy unless progesterone is administered to the female (Encyclopedia Britannica, 2009).

2.5 Intrauterine programming of Physiological systems

The intrauterine conditions in which the mammalian fetus develops have an important role in regulating the function of its physiological systems later in life. Changes in the intrauterine availability of nutrients, oxygen, and hormones program tissue development and lead to abnormalities in adult cardiovascular and metabolic function in several species. The timing, duration, severity, and type of insult during development determines the specific physiological outcome. Intrauterine programming of physiological systems occurs at the gene, cell, tissue, organ, and system levels and causes permanent structural and functional changes, which can lead to overt disease, particularly with increasing age.

2.6 Mechanisms of Intrauterine Programming

Intrauterine programming can occur at any level within the affected physiological system and may involve structural and functional changes in genes, cells, tissues, and even whole organs. These changes may be isolated or widespread events with either discrete or cumulative effects on

development depending on the nature and timing of the programming stimulus (McMillen and Robinson, 2005).

Epidemiological studies have shown that impaired intrauterine growth is mostly associated with increased incidences of cardiovascular, metabolic, and other diseases in later life (Barker, 1994b). Low birth weight particularly has been linked by various authors to hypertension, ischemic heart disease, glucose intolerance, insulin resistance, type 2 diabetes, hyperlipidemia, hypercortisolemia, obesity, obstructive pulmonary disease, and reproductive disorders in the adult. These associations have been described in populations of different age, sex, and ethnic origin (Baker, 1985, Gluckman and Hanson, 2005). Similarly morphometric analyses of the human epidemiological data have shown that certain patterns of intrauterine growth are related to specific adult diseases. Thin infant with the low ponderal index, rather than the symmetrically small baby, have been shown to be more prone to type II diabetes as adult (Phillips, 1993). These observations among others have led to the concept that adult disease originates in utero as a result of changes in development during suboptimal intrauterine conditions often associated with impaired fetal growth (Barker, 1994a). The process by which early insults at critical stages of development lead to permanent changes in tissue structure and function is known as intrauterine programming (Lucas, 1991).

2.7 Adult diseases associated with suboptimal intrauterine conditions in humans

Several experimental techniques in a number of species that compromises the intra uterine environment have been used to demonstrate intra uterine programming (Mcmillen and Robinson, 2005). Induction of intrauterine growth retardation (IUGR) by maternal stress, hypoxia, glucocorticoid administration, dietary manipulation, or placental insufficiency have been

reported to lead to postnatal abnormalities in cardiovascular, metabolic, and endocrine function in rats, guinea pigs, sheep, pigs, horses, and primates (Fowden *et al*., 2005,Mcmillen and Robinson, 2005). Similarly, in naturally occurring IUGR in polytocous species, low birth weight have been reported to be associated with postnatal hypertension, glucose intolerance, and alterations in the functioning of a number of endocrine axes, including the pancreatic islets, renin-angiotensin system, and hypothalamic-pituitary-adrenal (HPA) axis (Fowden, 1995). Animal studies have also demonstrated that the timing, duration, and exact nature of the insult during pregnancy are important determinants of the pattern of intrauterine growth and the specific physiological outcomes (Bertram and Hanson, 2001). Furthermore, these studies have shown that maternal insults with little, if any, effect on birth weight can alter subsequent cardiovascular and metabolic function (Barker, 2001). Together, animal experiments and human epidemiological data have shown that a wide range of individual tissues and whole organ systems can be programmed in utero with adverse consequences for their physiological function later in life. This programming occurs across the normal range of birth weights with worst prognoses for the extremes (Barker, 1994b and Mcmillen and Robinson, 2005).

2.8 Causes of Intrauterine Programming

Fowden *et al*., (2006) have asserted that in mammals, programming by internal and external cues during early postnatal life has been well established. According to them also, the concept that the adult phenotype also depends on environmental signals operating during intrauterine development has also received most attention. Since fetal growth and development depends primarily on nutrient and oxygen supply (Harding and Johnson, 1995), the associations between low birth weight and adult phenotype have been linked to poor nutrition and oxygenation during

early life. However, since nutrient and oxygen availability invariably affect the endocrine environment, the role of hormones as programming signals has also been examined in humans and experimental animals (Fowden and Forhead, 2004).

2.9 Studies on Programming by manipulating Nutrition

According to Fowden *et al*., (2006), prenatal nutritional programming of postnatal physiological functions have been demonstrated experimentally in a wide variety of laboratory and farm animals by manipulating the availability of macronutrients and micronutrients during development. Different techniques have been used to reduce the fetal availability of macronutrients used for tissue accretion (Bertram and Hanson, 2001). These include calorie restriction, iso-calorific protein deprivation, placental restriction, and reductions in umbilical and/or uterine blood flows. All of these procedures impair fetal growth and lead to abnormalities in cardiovascular, metabolic, and endocrine function both before and after birth. Altered postnatal physiological function has also been observed after maternal fat feeding and dietary manipulation of specific micronutrients, such as minerals (calcium, iron), co-factors (folic acid, taurine), and vitamins (A and D) (Armitage *et al* 2004). In some instances, the programming effects of macronutrient restriction were ameliorated by supplementation of the altered diet with single cofactors and amino acids (Armitage *et al* 2004). Indeed, it may be the balance of micro- and macronutrients that is more important in programming than the absolute amount of nutrient per se according to Fowden *et al*., (2006).

2.10 Studies on Programming by manipulating oxygenation

Several techniques have been used to induce fetal under-nutrition and also reduce fetal oxygen delivery. Chang *et al*., (1984), and De Grauw *et al*., (1986) have reported that in rats, chronic

hypoxia during pregnancy resulted in disproportionate IUGR and led to abnormalities in cardiovascular function in the adult offspring. Similarly, in human populations, the hypobaric hypoxia of high altitude has been linked with reduced birth weight and asymmetric growth retardation (Giussani *et al*., 2001, Moore *et al*., 2004). However, the developmental changes induced by hypoxia may also have nutritional component according to De Grauw *et al*., (1986), Giussani *et al*., (2001) because high-altitude populations are often impoverished and chronic hypoxia during pregnancy in experimental animals reduces maternal food intake (De Grauw *et al*., 1986, Giussani *et al*., 2001). The relative contributions of fetal hypoxemia and under-nutrition to programming in these circumstances have been investigated experimentally by pair-feeding pregnant rats at normal PO_2 and by using chick embryos. In avian species, specific developmental effects of oxygen ($O_{2)}$ deprivation can be studied in vivo without the confounding effects of a hypoxic placenta and/or reduced maternal dietary intake (Ruijtenbeek *et al*., 2003). Both of these approaches showed that hypoxia alone during early development can program the cardiovascular system and, more specifically, the blood vessels. In the chick embryo, hypoxia during the mid to late incubation period was found to exaggerate responses to peri-arterial sympathetic nerve stimulation and down-regulates nitric oxide-dependent dilator function in the vessels of the adult birds. In rats, prenatal hypoxia but not nutrient restriction was also found to impair endothelium-dependent relaxation in the mesenteric circulation of the adult offspring (Williams *et al*., 2005).

2.11 Studies on programming by manipulating Endocrine axis

Hormones play a central role in regulating normal growth and development in utero. Their concentrations and bioactivity have been reported to change in response to many of the

environmental challenges known to cause intrauterine programming (Fowden and Forhead, 2004). Under-nutrition, hypoxemia, and stress can alter both maternal and fetal concentrations of many hormones including growth hormone (GH), insulin-like growth factors (IGFs), insulin, glucocorticoids, catecholamines, leptin, thyroid hormones, and placental hormones such as the eicosanoids, sex steroids, and placental lactogen. Since some of these hormones cross the placenta, the fetal endocrine response to adverse conditions reflects the activity of both maternal and fetal endocrine glands and depends on the type, duration, severity, and gestational age at onset of the insult. In general, suboptimal intrauterine conditions lower anabolic hormone levels and increase catabolic hormone concentrations in the fetus (Fowden, 1995). These endocrine changes then affect fetal growth and development either directly or indirectly by altering the delivery, uptake, and metabolic fate of nutrients in the feto-placental tissues (Fowden, 1995). Direct manipulation of glucocorticoid, androgen, and thyroid hormone levels in utero have also been reported to alter fetal development and have long term consequences for cardiovascular, reproductive, and metabolic function (Fowden *et al*., 1998, Rhind *et al*., 2001).

Of the hormones known to regulate fetal development, glucocorticoids have been reported as the most likely to have widespread programming effects in utero (Bertram and Hanson, 2002 and Seckl, 2004). Glucocorticoids are growth inhibitors and affect development of all the tissues and organ systems that are at increased risk of adult pathophysiology when fetal growth is impaired (Fowden *et al*., 1998). Studies by Bertram and Hanson, (2002), Matthews,(2000), Seckl, (2004) in rats, guinea pigs, and sheep, have found out that fetal over-exposure to either endogenous or exogenous glucocorticoids leads to hypertension, glucose intolerance, and abnormalities in HPA function after birth. The specific postnatal effects of these treatments were found to not depend only on the gestational age at onset and the duration of exposure but also on the sex of the

offspring. In addition, programming effects of under-nutrition was reported to be preventable by abolishing maternal glucocorticoid synthesis by adrenalectomy or metyrapone treatment (Langley-Evans, 2001). It was concluded therefore that glucocorticoid program tissues in utero and may also mediate the programming effects of nutritional and other environmental challenges during pregnancy (Fowden and Forhead, 2004).

2.12 Critical Periods of Intrauterine Programming due to environmental assaults

Programming can be caused by environmental insults at many stages during development. During the peri-conceptual and pre-implantation periods, nutrient, O_2, and hormone levels affect development of the oocyte and blastocyst, with consequences for the distribution of cells between the trophoblast and inner cell mass. Exposure to excess progesterone and metabolites, such as urea, specifically during these periods can lead to enhanced birth weight in sheep and pigs as reported by McEvoy *et al*., (2001). Dietary restrictions during the peri-conceptional period have also been shown to shorten gestation and cause hypertension and abnormal HPA function in adult sheep (Kumarasamy *et al*., 2005). Developmental changes arising before implantation are likely to affect many cell lineages, although adaptations later in gestation, such as up-regulation of placental nutrient and O_2 transport, may compensate for the early defects and normalize birth weight. Once placentation has begun, programming effects of environmental signals may be mediated via changes in placental development (Godfrey, 2002). However, the actual extent to which early insults program the placenta per se remains unknown.

2.13 Critical Periods of intrauterine programming in rodents and humans

During organogenesis, environmental insults have been found to cause discrete structural defects that permanently reduce the functional capacity of the organ. Rhind *et al*., (2001) have reported

that when insult occurred during gametogenesis, reproductive potential of the next generation may be impaired. In the phase of rapid fetal growth, insults, which alter the supply, uptake, and utilization of nutrients, will influence tissue growth and may switch the cell cycle from proliferation to differentiation with adverse consequences for total cell number (Harding and Johnson ,1995, Fowden *et al*., 1998). Different fetal organs grow at different rates and the timing of the insult is important in determining the tissue specificity of the programmed effects. In late gestation, there is a period of fetal maturation during which many tissues undergo structural and functional changes in preparation for extra uterine life ((Fowden *et al*., 1998). Virtually all these processes are often glucocorticoid dependent and can be activated prematurely by early glucocorticoid exposure. In addition, birth itself activates physiological systems that have little or no function in utero but are essential for neonatal viability, such as ventilation, thermoregulation, gluconeogenesis, enteral nutrition, and appetite control (Fowden *et al*., 1998). Changes in prepartum maturation in relation to the timing of delivery may, therefore, have consequences for the set point and sensitivity of key physiological systems during the perinatal period, which can then persist, revert, or amplify in later life (Fowden *et al*., 2006).

Although the sequence of developmental changes is broadly similar in all mammalian species, differences do exist in their precise timing between animals. In altricial species that are immature at birth (e.g., rodents and rabbits), several of the physiological systems known to be programmed in utero continue to develop after birth. The period of developmental plasticity, therefore, extends after birth in these species in contrast to precocial species (e.g., human, sheep, and pig) that are more physiologically mature at birth ((Fowden *et al*., 1998). According to studies changes in nutrient availability, hormone concentrations, and maternal behavior during the immediate neonatal period in rats have been found to alter their subsequent cognitive, neuro-

endocrine, and reproductive function (Ozanne and Hales, 2002, Weaver *et al*., 2004, Ross and Desai, 2005). Consequently, postnatal environmental changes, particularly before weaning, may ameliorate or exaggerate the morphological and functional changes programmed in utero (Ross and Desai, 2005).

2.14 Relevance of gene in programming

Young, (2001) opined that the associations between birth weight and later risk of degenerative disease may be due to a direct genetic link between intrauterine growth and disease susceptibility inherited at conception. This partly reflects the survival value of genes selected for reduced fetal growth yet rapid postnatal growth and fuel storage during evolution in populations subjected to periodic under-nutrition (Bateson *et al*., 2004, Ross and Desai, 2005). The observation that mutations in the pancreatic glucokinase gene result in reduced fetal insulin secretion, lower birth weight, and adult glucose intolerance indicates that fetal growth and disease susceptibility can be linked through a single gene (Hattersley and Tooke, 1999). However, these monogenic disorders are rare and cannot explain the variation in disease risk across the normal birth weight range. In populations like the Pima Indians who have a high incidence of type 2 diabetes, there is evidence for both a genetic and an intrauterine origin of the relationship between birth weight and adult insulin resistance (Frayling and Hattersley, 2001). The discordant disease risks in monozygotic twins of discrepant birth weights compared with the concordant risks when birth weight is similar also shows that different adult phenotypes can develop from the same genotype when intrauterine growth is compromised (Phillips ,1993). However, different specific polymorphisms of genes involved in growth and metabolism, such as *Igf1*, *PPARγ*, and the *VNTR* region of the insulin gene, may alter susceptibility to environmental signals and account for some of the genetic variation inherent in intrauterine programming (Moritz *et al*., 2003).

During development, DNA can be modified epigenetically to alter gene expression without a change in DNA sequence (Vickaryous and Whitelaw, 2005). In mammals, these modifications occur primarily by DNA methylation and/or posttranslational alterations to the histones packaging the chromatin. In turn, these conformational changes alter the interaction between DNA and its regulatory proteins at the promoters, with effects on gene activation and repression (Waterland and Carza, 1999). DNA methylation is particularly important in genomic imprinting, the process by which one allele of a gene is silenced in a parent-of-origin manner (Constancia *et al*., 2004). Imprinting has a major role in early mammalian development, and many of the 80 known imprinted genes are involved in controlling placental and/or fetal growth (Reik *et al*., 2003, Constancia *et al*., 2004). Epigenetics, therefore, provides a molecular mechanism for programming that links genes, the prenatal environment, intrauterine growth, and subsequent susceptibility to disease.

Two main periods of epigenetic modification as identified by Vickaryous and Whitelaw, (2005) are gametogenesis and early embryogenesis. These are the times of widespread demethylation and resetting of the epigenetic marks by de novo methylation. They are, therefore, particularly vulnerable to changes in the availability of methyl donors and cofactors essential for one-carbon metabolism, such as folate and glutathione (Waterland and Carza, 1999). Once established, the epigenetic marks are stable mitotically and create unique, lineage-specific patterns of gene expression and/or silencing. Epigenetic modifications can also occur later in development, as changes in DNA methylation and imprint status have been observed during the perinatal period in several species (Waterland and Carza, 1999, Constancia *et al*, 2004). For example, in ovine and human liver *Igf2* imprinting switches from monoallelic expression in the fetus to biallelic

expression in the adult, whereas in rodents *Igf2* is completely silenced in all somatic tissues at weaning (Fowden, 2003).

Both nutritional and endocrine factors have been shown to influence the epigenotype (Waterland and Jirtle, 2004). Glucocorticoid administration have also been found to cause DNA demethylation associated with increased gene expression of a hepatic aminotransferase in rats during the perinatal period (Thomassin *et al*., 2001). In cultured pre-implantation sheep and mouse embryos, the amino acid composition of the culture medium was found to alter the methylation state of key imprinted genes and change intrauterine growth after embryo transfer (Young, 2001). Similarly, in neonatal rat kidney, utero-placental insufficiency induced by uterine artery ligation during late gestation was found to cause hypo-methylation of *p53*, a gene involved in apoptosis (Pham *et al*., 2003). When methyl donor availability is altered by a low-protein diet during pregnancy, the pattern of hepatic DNA methylation was found to change in both the fetus and adult offspring (Rees *et al*., 2000, Lillycrop *et al*., 2005). Addition of folate or methyl donors, such as glycine and taurine, to the low-protein diet restores normal patterns of DNA methylation and prevents the abnormalities in adult cardiovascular function that are programmed by the un-supplemented diet (Armitage *et al*.,2004).

Environmental challenges may, therefore, cause programming by altering gene expression via several mechanisms. These include loss of imprinting, differential promoter usage, and up- or down-regulation of expression from the active allele(s) mediated epigenetically or via transcription factors (Waterland and Carza, 1999). Definitely, in several genes with multiple mRNA transcripts, the relative abundance of the different splice variants is altered by under-

nutrition and glucocorticoid exposure in a tissue-specific manner, with potential consequences for protein translation in fetal tissues (Fowden, 2003).

2.15 Cells and programming

Changes in transcription induced by environmental challenges have been found to lead to altered protein synthesis and permanent changes in cellular protein abundance (Fowden *et al*., 2006). These proteins include receptors, ion channels, transporters, enzymes, growth factors, cyto-architectural proteins, binding proteins, and components of several intracellular signaling pathways. In particular, there are changes in the adenylyl cyclase and insulin-signaling pathways in cells, such as adipocytes, hepatocytes, and myocytes, in response to nutrient and O_2 deprivation, which persist after birth and influence adult metabolic activity (Ozanne and Hales, 2002, McMillen and Robinson, 2005). Similarly, prenatal under-nutrition and hypoxemia was hinted to induce permanent changes in the abundance of membrane and nuclear receptors for several hormones including glucocorticoids, catecholamines, insulin, IGFs, GH, leptin, and prolactin (Bertram and Hanson, 2002, Fowden and Forhead, 2004, Fowden *et al* 2005, McMillen and Robinson, 2005). Overall, these changes in protein synthesis alter cell metabolism and growth and modify sensitivity to subsequent challenges.

2.16 Cellular processes that may be programmed by intrauterine manipulation of the nutritional or hormonal environment

1) Hormone receptors. 2) Intracellular signaling pathways. 3) Ion channels. 4) Transporters for nutrients (e.g., glucose or amino acids) or minerals. 5) Protein synthesis. 6) Enzyme activities by de novo synthesis or phosphorylation through intracellular signaling pathways and/or ion

channels. 7) Mitochondrial oxidative phosphorylation and thermogenic activity (Fowden *et al*., 2006).

Environmentally induced changes in cell physiology can occur at any point in development but are more likely to have long-term consequences during the formation of cell lineages and when cells are differentiating during late gestation in preparation for extra-uterine life. In addition, when cells are growing at their maximum rate, under-nutrition and hypoxemia may reduce cell proliferation directly by limiting substrate availability for tissue accretion and energy generation. Indeed, restriction of uterine blood flow near term is known to reduce DNA synthesis in fetal cells with a high proliferation rate in utero (Asano *et al*., 1997). Suboptimal intrauterine conditions may, therefore, induce changes in the function, number, and size of cells by altering proliferation, clonal selection, and apoptotic remodeling of cell populations (Fowden *et al*., 2006).

2.17 Changes in tissues and organs during programming

The changes in cell structure and function alter the morphology and physiology of tissues and organs as a whole. If the insult occurs at the time of organogenesis, the changes may be severe and lead to a permanent developmental deficit. For instance, glucocorticoid administration to pregnant ewes for 2 days when the mesonephric kidney is developing at the end of the first month of gestation was reported to cause a permanent reduction in nephron number and leads to hypertension in the adult offspring (Moritz *et al*, 2003). More subtle changes in cell composition of tissues, induced by suboptimal conditions in utero, can also influence postnatal physiological function. For example, there are changes in the relative proportions of different cell types in the pancreatic islets, liver, and skeletal muscles after IUGR that are associated with adult insulin

resistance, glucose intolerance, and hypertension (Ozanne and Hales, 2002, Holemans *et al*., 2003, McMillen and Robinson, 2005). In organs like the placenta, early changes in development at the gene (imprinting), cell (transporter abundance), and tissue (vascularity) levels have been reported to impair fetal nutrient and O_2 delivery throughout gestation with implications for tissue programming long after the original insult (Godfrey,2002 and Reik *et al*.,2003).

2.18 Changes in body systems during programming

The postnatal physiological abnormalities observed after suboptimal intrauterine conditions are multifactorial and involve several organs and endocrine systems .The severity of outcome is, therefore, determined by the number of organs and systems adversely affected by the intrauterine insult, which, in turn, depends on the nature and duration of the insult in relation to the stage of development (Gluckman and Hanson, 2005). In sheep for instance, maternal glucocorticoid treatment early in gestation leads to hypertension but not glucose intolerance, whereas treatment late in gestation has the opposite effects in the adult offspring (Moritz *et al*, 2003 and Fowden and Forhead, 2004). In some systems, a specific trigger, or a second challenge, is required postnatally to unmask the intrauterine programming. In pancreatic islets, the abnormalities in insulin secretion induced by mild prenatal under-nutrition only become evident in the adult when the demand for insulin rises during pregnancy (Holemans *et al*., 2003). Similarly, in the heart, an ischemic challenge in adulthood is required to expose the underlying abnormalities in development induced by prenatal hypoxia or cocaine exposure (Li *et al*., 2003). In several physiological systems, sex-linked differences in intrauterine programming do not appear until puberty when the onset of gonadal steroidogenesis uncovers physiological abnormalities in peripheral tissues or in the hypothalamic-pituitary-gonadal axis itself (Matthews, 2000, Rhind *et al*., 2001). However, in the majority of physiological systems studied, the adverse consequences

of intrauterine compromise become more evident with increasing age as compensatory adaptations in other tissues and organ systems fail (McMillen and Robinson, 2005).

2.19 Consequences of Intrauterine Programming

The consequences of intrauterine programming depend on whether the developmental deficit is the inadvertent outcome of an insult acting as mutagen or a specific adaptation to an environmental challenge designed to maximize survival to reproductive age (Gluckman *et al*., 2005). In mutagenesis, the structural and functional deficits are permanent and invariably detrimental to long-term survival. By contrast, the physiological adaptations made in response to suboptimal intrauterine conditions may improve viability in the short to medium term but at the risk of later morbidity (Hanson and Gluckman, 2005). The adaptations in functional capacity programmed in utero may, therefore, be a trade-off to maintain development of essential tissues, such as the brain and placenta, and/or a mechanism for setting an appropriate phenotype for the environmental conditions ex utero (Bateson *et al* 2004, McMillen and Robinson, 2005). When the functional capacity set in utero does not match that required postnatally, homeostasis may be compromised and lead to abnormal physiological parameters, which, eventually, result in overt disease. For example, the enhanced sensitivity of the HPA axis programmed by suboptimal conditions in utero is essential for survival in poor conditions but is inappropriate when nutrients and oxygen are plentiful and may cause hypertension and metabolic disorders in the adult (Fowden *et al*., 2005). Similarly, intrauterine programming of a thrifty phenotype by prenatal under-nutrition will lead to increased growth and fat deposition if postnatal nutrient availability is better than predicted in utero (Hales and Barker, 2001, Ozanne and Hales, 2002, Symonds *et al*., 2003). In turn, the increased adiposity will lead to adult insulin resistance, glucose intolerance, and finally type 2 diabetes, particularly as the counter-regulatory mechanisms

deteriorate with age (Kelly and Trasler, 2004). In humans, the risk of developing adult type 2 diabetes is greatest when poor prenatal growth is coupled with catch-up growth and adiposity rebound during childhood (Gluckman and Hanson, 2005). Indeed, the accelerated growth often observed after IUGR may be more important than the intrauterine changes per se in programming adult-onset degenerative diseases (Singhal and Lucas, 2005).

Intrauterine programming also has consequences for the next generation. The physiological perturbations programmed by environmental challenges in utero can be transmitted from one generation to the next by either the mother or father (Drake and Walker, 2004, Ross and Desai, 2005). This trans-generational inheritance of programming may be due to changes in the primordial germ cells from which the next generation develops or to recapitulation of the metabolic and endocrine conditions that the mother experienced as a fetus in utero due to the programmed adaptations in her physiology (Aerts and Van Assche, 2003, Kelly and Trasler, 2004). Alternatively, it may reflect meiotic inheritance of the epigenetic marks as a result of aberrant demethylation (Young, 2001, Vickaryous and Whitelaw, 2005). Incomplete erasure of the epigenetic marks may lead to an inheritable "memory" of epigenetic state at specific alleles.

Since nutrition has improved progressively in most developed and developing countries over the past 50 years, many people will have developed a phenotype in utero that is programmed for a significantly lower level of nutrition than currently available. This may explain, in part, the epidemic of hypertension, obesity, and type 2 diabetes in populations worldwide. As food intake, lifestyle, and dietary advice during pregnancy change in response to these trends, the physiological adaptations now being programmed in utero by Western high-fat/high-calorie diets may lead to adult phenotypes no better suited to the prevailing environmental conditions in the

next generation than those programmed by poor nutrition a generation ago. However, improved knowledge of the pathophysiology induced by transgenerational oscillations in dietary intake and other environmental factors may enable the development of nutritional and other interventions to ensure that intrauterine programming is beneficial and not detrimental to adult health (Fowden *et al*., 2006).

2.20 *Ricinus communis* plant.

The castor oil plant (*Ricinus communis L.*) is a member of the spurge family of plants (Euphorbiaceae). It is grown commercially for the oil contained in the seed, which is used primarily for industrial purposes and in the manufacture of cosmetics. Most of the world's castor oil is produced in India, China and Brazil, but commercial production also occurs on a smaller scale in many other tropical countries(EFSA, 2008). Production of castor oil increased from 0.4 million tonnes in 1970 to 0.8 million tonnes in 2000 (Weiss, 2000).

The plant is known by several names including; English: Castor Bean, castor oil plant, Hindi: Endi, Sanskrit: Eranda. Locally the plant is known in Nigeria by such names as “Zurman” (Hausa), “Laraa” (Yoruba). “Ogilisi” (Igbo), “Kpamfini gulu” (Nupe), “Jongo” (Tiv), and “Era ogi” (Bini) (Jumbo and Emenebiaku, 2008). The plant has been in cultivation for well over 6000 years. Greek physicians of the first century AD regarded the oil as suitable only for external application, a view which persisted until the 18th century, when it was listed in many pharmacopoeias as a purgative. The generic name is from the Latin ricinus, meaning 'tick', because the mottled seeds of the plant are similar in shape to these insects.

The oil makes up about 50% of the weight of the seeds. The oil is mostly constituted of ricinoleic acid, with small amounts of dihydroxystearic, linoleic, oleic, and stearic acids. Uses of Castor oil include manufacture of pharmaceuticals and cosmetics and as a laxative (Cosmetic Ingredient

Review Expert Panel, 2007), in the textile and leather industries, and for manufacturing plastics, fibres soaps, printing inks, wetting agents, and lubricants (Weiss, 2000).

2.21 Botanical description *Ricinus communis* plant

It is a tall, branched shrub, reaching up to 4 m in height. The stem is erect and hollow, greyish-green when young and becoming brownish-red when older. The leaves are petioled, green and occasionally frosted blue or red, and arranged in a spiral. The blade is usually divided into palmate, ovate-oblong lobes of up to 60 cm in diameter. The ribs are palmate and the margins irregularly serrate. The inflorescences are terminal panicles 15-50 cm long, with the female flowers in the upper section of the inflorescence. The perianth is divided into five lobes and the style has three red, doubly split stigma branches. The male flowers bear numerous, heavily branched stamens with up to 1000 separate bursting anthers. The fruit capsule is soft and prickly or smooth and grooved, 1-2.5 cm in diameter. The capsule bursts open when ripe, showing the large, brightly speckled seeds (Duke and Wain, 1981).

2.22 Bioactive compounds in *Ricinus communis* plant seed

The castor plant contains in its seeds a group of closely related glycoproteins (the ricin group), ricinoleic acid (12-hydroxyoleic acid) and the alkaloid ricinin. The latter compound is often used as an indicator of the presence of material from castor beans in press cakes in feeding stuffs (Darby *et al.*, 2001).The seed oil was, especially earlier, used as a laxative/purgative due to the direct effect of the ricinoleic acid on the small intestine. The free acid is released by enzymatic hydrolysis in the intestines from the lipids in which is makes up around 70% of the fatty acid residues (Hänsel and Hass, 1983). The toxicity of *Ricinus* seeds has been recognised since ancient times, and its toxicity to humans has recently been reviewed (Olsnes, 2004; Audi *et al.*,

2005). Castor seeds were used in the classical Egyptian and Greek medicine, and were described in the Sanskrit work on medicine, *Susruta Ayurveda* from the sixth century B.C. (Olsnes, 2004). Intoxication by castor beans is not rare in countries where the plant grows in abundance, and by 1974 at least 700 cases of human intoxication had been described (Balint, 1974). More than a century ago, Stillmark isolated a toxic protein from the seeds, which he termed ricin (Stillmark, 1888). At that time the toxicity was believed to result from the observed ability of the ricin preparation to agglutinate blood cells. More recent studies have established that Stillmark's ricin preparations were a mixture of two proteins, namely the potent cytotoxin (ricin) and a haemagglutinin (Lord *et al*., 1994).

2.23 CHEMISTRY OF *RICINUS COMMUNIS* OIL

2.24 Definition and Structure

According to the International Cosmetic Ingredient Dictionary and Handbook (Gottschalck and McEwen 2004), *Ricinus communis* (Castor) Seed Oil is defined as the fixed oil that is obtained from the seeds of *Ricinus communis*. Other names for this oil include: Castor Oil, Castor Oil (*Ricinus communis* L.), Castor Seed Oil, *Ricinus communis*, and *Ricinus communis* Oil (Gottschalck and McEwen 2004), and *Ricinus* Oil (TNO BIBRA International Ltd. 1999).

As given by TNOBIBRA International Ltd. (1999), the structural formula is:

CH2OR
|
CHOR
|
CH2OR

Where R represents a fatty acyl group [CH3 (CH2)5CH (OH)

CH2CH=CH (CH2)7COOH] that is typically derived from ricinoleic acid. Ricinoleic acid accounts for 87% to 90% of the fatty acyl groups, and the following other fatty acids comprise the remaining fatty acyl groups: oleic acid (2% to 7%), linoleic acid (3% to 5%), palmitic acid (1% to 2%), stearic acid (1%), dihydrostearic acid (1%), and trace amounts of other fatty acyl groups (not specified) (TNO BIBRA International Ltd. 1999). According to other sources, Castor Oil contains 2.4% lauric acid (Larsen *et al*. 2001), 2 to 5% linoleic acid, (Maier *et al*., 1999), and globulin, cholesterol, lipase, vitamin E, and *β*-sitosterol (Scarpa and Guerci, 1982).

2.25 Chemical and Physical Properties of *Ricinus communis* oil:

Property	Value	Reference
	Castor Oil	
Color/Form	Colorless to pale-yellow viscous liquid	Lewis 2000
Taste	Slightly acrid	National Toxicology Program (NTP) 2003
Specific gravity	0.945 to 0.965 at 25°/25°C; 0.961 to 0.963 at 15.5°/15.5°C	NTP 2003
Density	0.953 to 0.965 g/ml at 20°C	NTP 2003
Viscosity	6 to 8 poises at 25°C	NTP 2003
	283 cP at 37°C	Fredholt et al. 2000
Solubility	In water (<1 mg/ml at 20°C); in DMSO (≥100 mg/ml at 20°C); In 95% ethanol (≥ 100 mg/ml at 20°C); in methanol (miscible); In acetone (≥100 mg/ml at 20°C)	NTP 2003
	Miscible in absolute alcohol, glacial acetic acid, chloroform, and ether	Lewis 2000
Surface tension	39.0 dynes/cm at 20°C; 35.2 dynes/cm at 80°C	NTP 2003
Refractive index	1.4784 at 20°C; 1.473 to 1.477 at 25°C; 1.466 to 1.473 at 40°C	NTP 2003
Optical rotation	Not less than +3.5°	NTP 2003
Flash point	229°C (445°F)	NTP 2003
Autoignition temperature	448°C (840°F)	NTP 2003
Melting point	−12°C	NTP 2003
Boiling point	313°C	NTP 2003
Freezing point	−10°C	NTP 2003
Saponification value	178	NTP 2003
Iodine value	85	NTP 2003
Acid value	<4	NTP 2003
Reichert-Meissl value	<0.5	NTP 2003
Polenske value	<0.5	NTP 2003
Acetyl value	144 to 150	NTP 2003
Hydroxyl value	161 to 169	NTP 2003

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2.26 Methods of Production of *Ricinus communis* oil

According to Kathren *et al*., (1959), castor oil is extracted from the bean of the tropical plant, *Ricinus communis*, by either of the following two methods: (1) use of a solvent and (2) mechanical crushing, grinding, and pressing. The former method is more efficient, leaving a more dessicated residue. This residue, known as pomace, contains ricin and a separate allergen (Ratner and Gruehl, 1929). Following extraction from the castor bean, neither of these two

fractions is present in castor oil (Ordman, 1955). According to a more recent reference (Cornell University, 2001), ricin does not partition into the oil because of its water solubility. Therefore, castor oil does not normally contain ricin, provided that cross-contamination does not occur during its production. Other sources indicated that castor oil is produced via the cold pressing of the seeds of *Ricinus communis* (Hui, 1996) and by cold expression and subsequent clarification of the oil by heat (Gennaro, 1990).

2.27 Analytical Methods on Castor Oil

Castor oil has been analyzed using the following methods: mass spectroscopy (Ayorinde *et al*. 2000), gas-liquid chromatography (Kato and Yamaura, 1970; Ramsey *et al*., 1980), thin-layer chromatography (Srinivasulu and Mahapatra, 1973), and non-aqueous reverse-phase high-performance liquid chromatography–mass spectrometry (St¨ubiger *et al*., 2003).

2.28 Use of *Ricinus communis* oil in Cosmetics

Ricinus communis (Castor) Seed Oil functions as a fragrance ingredient and a skin-conditioning agent—occlusive in cosmetic products, and Hydrogenated Castor Oil functions as a skin-conditioning agent—occlusive and a viscosity increasing agent—non aqueous (Gottschalck and McEwen, 2004).

2.29 Extent of Use in Cosmetics

Frequency of use data based on industry reports to the Food and Drug Administration (FDA) in 2002 indicated that *Ricinus communis* (Castor) Seed Oil and Hydrogenated Castor Oil are being used in a total of 769 and 202 cosmetic products, respectively. Use concentration data also obtained from an industry survey by the Cosmetic, Toiletry, and Fragrance Association (CTFA,

2004) indicated that *Ricinus communis* (Castor) Seed Oil and Hydrogenated Castor Oil are being used in cosmetics at concentrations of up to 81% and 39%, respectively. Of the product categories, the highest reported used concentration for *Ricinus communis* (Castor) Seed Oil is associated with lipsticks. Two other sources indicated that lipstick contains 44% *w/w* castor oil (Hui, 1996) and 10% to 67% castor oil (Smolinske, 1992).

2.30 Non cosmetic use of *Ricinus communis* oil

According to Aplin and Eiseo (1997), castor oil is used as an industrial lubricant and as a medicinal purgative; the authors noted that medicinal Castor Oil does not contain ricin. Mc-Keon *et al*. (2000) confirmed lubricant and anti-fungal uses and additional uses in paints, coatings, and plastics. According to FDA's *OTC (Over-the-Counter) Drug Review Ingredient Status Report* (FDA 2003a), castor oil is classified as generally recognized as safe and effective for use as a stimulant laxative, but not generally recognized as safe and effective for use as a wart remover. For use as a stimulant laxative, the single daily dose reported for one product ranges from 15 to 60 ml for adults and children ≥12 years (Drugstore.com, Inc.2004). FDA had issued a proposed rule in 1982 stating that Castor oil is generally recognized as safe and effective and not misbranded when used as an active ingredient (laxative) in OTC laxative drug products (FDA, 1985). Castor oil is included in the list of inactive ingredients (excipients) present in approved oral, intramuscular, and topical drug products or conditionally approved drug products that are currently marketed for human use (FDA, 2003b).

The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (1980) established an acceptable daily intake (for man) of 0 to 0.7

mg/kg body weight for castor oil. The determination of range was based on consideration of the lack of adequate long-term studies.

2.31 Biological properties of *Ricinus communis* oil

2.32 Absorption, Distribution, Metabolism, and Excretion of *Ricinus communis* oil:

In a study by Paul and McKay (1942), using rabbits (weight= 3 kg) and fed 6% castor oil in the diet for 18 days with fecal collection during the last ten days, utilization (uncorrected for metabolic fat) of castor oil was found to be 92.1%, which the authors considered to be efficient utilization, while the percentage of fat in the feces was 2.2%.

Watson and Gordon (1962) studied the digestion, absorption, and metabolism of castor oil (medicinal grade) in eight male Sprague-Dawley rats (weights=100 to 200 g). The composition of the castor oil used was ricinoleic acid (90.0%), linoleic acid (4.7%), oleic acid (3.2%), stearic acid (1.0%), palmitic acid (1.0%), and palmitoleic acid (0.1%). In the first experiment, four rats were fed rat chow ad libitum, and the remaining four were fasted overnight. On the following morning, castor oil (1.0 ml) was dosed via stomach tube and chyle was collected over a 24- h period. The mean values for percent recovery of ricinoleic acid in fasted and fed rats were 6.8% and 24.2%, respectively ($p < .01$).

In the second experiment, seven weanling rats were fed a diet consisting of rat chow that had been mixed with castor oil (20% by weight). The control group was fed an olive oil-supplemented diet. After 4 and 8 weeks of feeding, an epididymal fat pad was removed from each rat in both groups, and fatty acid composition was determined using gas-liquid chromatography. Mean values for ricinoleic acid in the fat pad after 4 and 8 weeks of feeding were 9.1±1.7 and 9.7±1.0%, respectively. Ricinoleic Acid was undetectable in the fat pads of

control rats fed olive oil. For rats fed castor oil, random analyses of feces indicated a considerable fraction of hydroxystearic acid. However, hydroxystearic acid was undetectable in the feces of rats fed a normal diet. The authors suggested that hydrogenation of ricinoleic acid in the gut lumen by intestinal bacteria would be a likely explanation for this finding.

The relationship between dose and absorption of castor oil was studied in the final experiment. Polyethylene cannulae were inserted into the thoracic duct and duodenum of each of two rats, and the animals received 0.5N saline overnight. On the following morning, one rat received 0.2 ml and one rat received 0.6 ml of castor oil, and the rats were killed 45 min post dosing. Only the high dose induced diarrhoea. Intestines (small and large) were excised, homogenized, and tissue lipids were extracted. Chyle was also collected and extracted. Total lipid estimation and gas-liquid chromatography (GLC) analysis of fatty acids were performed on the extracts. For the rat dosed with 0.2 ml castor oil, the percentage of ricinoleic acid in the chyle was 18.1% and the percentage detected in the small bowel lipids was 6.2%. For the rat dosed with 0.6 ml Castor Oil, the percentage of ricinoleic acid in the chyle was 3.0% and the percentage in the small bowel was 3.1%. Ricinoleic acid was undetectable in large bowel lipids from the rat with diarrhea. The results from the study indicated a close correlation between the dose administered and the percentage of ricinoleic acid in the faeces, i.e., greater absorption at the lower dose (Watson and Gordon, 1962).

Castor oil is metabolized to ricinoleic acid by pancreatic lipase in hamsters (Gaginella and Bass, 1978). Thompson (1980) reported that Castor Oil is a triglyceride that is hydrolyzed in the small intestine in humans by pancreatic enzymes, leading to the release of glycerol and ricinoleic acid.

2.33 Effect of Castor Oil on induction of labour and Prostaglandin Synthesis:

Gao *et al*., (1999) evaluated the effect of castor oil in the diet on the synthesis of prostaglandin E2 (PGE2) and the induction of labor using two groups of eight pregnant Wistar rats (at gestation day 18; test and control groups, respectively). Two milliliters of castor oil–containing diet were administered by gavage daily for a total of four feedings. The diet (labor-inducing diet) consisted of castor oil (30 ml) + one chicken egg, blended and heated to a thick consistency. At 4 h after the fourth feeding, the pregnant females were killed. Compared to the control group, a significant increase in concentrations of PGE2 in tissues of the intestinal mucosa, placenta, amnion, and amniotic cells was noted in test animals. The authors stated that the increased synthesis of PGE2 "is a key of" the initiation of labor that is induced by a castor oil diet.

2.34 Reproductive and developmental toxicity effects of *Ricinus communis* oil

In a report by National Toxicological Programme, NTP (1992), groups of rats and mice were fed diets containing 0.62%, 1.25%, 2.5%, 5.0%, and 10% castor oil, respectively, continuously for 13 weeks. A decrease in epididymal weight (6% to 7%) was observed in mid- and high-dose groups of male rats. No effects on any other male reproductive end point (testes weight and epididymal sperm motility, density, or testicular spermatid head count) or female reproductive endpoint (estrous cycle length, or time spent in each phase of the cycle) were noted. Microscopically, there were no treatment-related lesions in any organ or tissue. These results indicated that there was little or no evidence of any reproductive toxicity in rats that was associated with castor oil in the diet. For male and female mice, castor oil in the diet had no adverse effects on any male or female reproductive parameter.

On the other hand Litvinova and Fedorchenko (1994) evaluated the influence of single doses of different plant oils on the estrous cycle and fertility using female Wistar rats. Castor oil (0.2 ml,

single dose) was injected intramuscularly on the first day after estrus. Study of the estrous cycle was based on the following: the cytologic picture that was obtained from vaginal smears, the phase structure (proestrus, estrus, metaestrus, and diestrus) of the cycle, and the frequency of estrus over a 15-day observation period prior to injection of castor oil (control) and a similar length of time after injection (test). Fertility was studied using intact animals (control) and animals that were treated with castor oil. On day 2, after castor oil injection (i.e., during diestrus), the females were mated with male rats. Control females were also mated with male rats on day 2. The first day of pregnancy was defined by the presence of spermatozoa in vaginal smears. Pregnant females were killed on day 20. The estrous cycle phases were recorded 3 to 4 times during the 15-day observation period.

The injection of castor oil was found to result in a reduction in the frequency of estrous by a factor of 1.4 and lengthening of the interestrous phase by 39.6%. The authors concluded that the latter finding may be indicative of prolongation of luteolytic processes and an inhibition of folliculogenesis.

In the female fertility experiments, mating occurred over a period of 3.22 ± 0.12 days. The index of fertility was calculated from the ratio of the number of mated females to the total number of females in the test. The fertility index for intact animals (control) was 100%, and pregnancy occurred in 100% of the cases. A decrease in the pregnancy index was reported for female rats injected intramuscularly with *Ricinus communis* oil. Compared to controls, *Ricinus communis* oil also caused a reduction in the number of corpora lutea in the ovaries, and this observation was said to have been a consequence of the suppression of folliculogenesis and ovulation. Additionally, the number of implantation sites in uteri from female rats injected with castor oil was decreased when compared to control rats. A decrease in the number of live fetuses in uteri

and an increase in pre- and postimplantation deaths was also observed in rats injected with castor oil. The authors concluded that castor oil injected intramuscularly, suppressed ovarian folliculogenesis and also had anti-implantation and abortive effects (Litvinova and Fedorchenko, 1994). Okwuasaba *et al*., (1991) evaluated anti-conceptive and estrogenic effects of a methanol extract of *Ricinus communis* var. *minor* seeds using adult albino Wistar rats (weights = 160 to 210 g), young albino Wistar rats (weights = 40 to 60 g), immature Swiss albino female mice (21 to 23 days old; weights = 10 to 13 g), and adult female New Zealand white rabbits (2.5 to 3.0 kg). In the anti-fertility experiment, adult female rats and rabbits with regular estrus cycles were used. The females were mated overnight (3:1 female/male ratio), and the day of mating was considered day 0. Castor bean extract was injected subcutaneously (s.c. dose of 0.6 or 1.2 g/kg) into female rats once daily for two days, and mating occurred on the third day. Female rabbits were injected intramuscularly with Castor bean extract (dose = 200 mg/kg). Control animals were injected s.c. with corn oil for two consecutive days. A laparotomy was performed to confirm that implantation had occurred. Castor bean extract (0.6 or 1.2 g/kg dose) prevented nidation in rats, and none of the treated rats delivered pups at term. The results for rabbits (200 mg/kg dose) paralleled those for rats. Nidation was prevented, and the extract protected the rabbits against pregnancy for over three gestation periods (i.e., 110 to 120 days). The normal gestation period for rabbits is 28 to 30 days. Effects induced by Castor bean extract were reversible in rats and rabbits. No fetal abnormalities were observed. In the study to determine estrogenic activity, the following endpoints were used to estimate the estrogenicity of the Castor bean extract: uterine weight ratio, degree of vaginal cornification, and quantal vaginal opening. In one experiment, groups of 10 Swiss albino female mice (21 to 23 days old; weights = 10 to 13 g) were injected s.c. with 1.0 or 4.0 mg/kg Castor bean extract (in corn oil, 0.2 ml) daily for 4

days. In a similar experiment, bilateral ovariectomy was performed on young, immature rats (groups of five), and the wound was sutured. After a 15-day recovery period, the groups of rats were injected s.c. with 0.6 or 1.2 g/kg Castor bean extract (in corn oil, 0.2 ml) daily for 4 days. In both experiments, suitable controls (injected s.c. with corn oil only) were maintained. Additionally, estradiol- 17β (reference hormone; dose = 10 μg/kg) was administered to rats (group of 10) and mice (group of 10). Mice and rats were necropsied 24 h after the last injection. Body and uterine wet weights were recorded. Results for bilaterally ovariectomized rats indicated that doses of 0.6 and 1.2 g/kg induced increases in relative uterine weight of 116.7 ± 6.5 ($p < .01$) and 211.6 ± 8.2 mg/100 g body weight ($p < .001$), respectively, compared to a control value of 83.3 mg/100 g body weight. Compared to rats, Castor bean extract (1.0 and 4.0 mg/kg) induced a qualitatively similar effect in immature mice. In rats and mice, the effect on relative uterine weight appeared to have been dose-dependent. Estradiol-17β also increased uterine weight in rats and mice. The relative uterine weights (mg/100 g body weight) for rats and mice dosed with estradiol-17β were reported as follows: 309.2 ± 11.4 (rats) and 517.5 ± 12.3 (mice). Cornification of the vaginal epithelium and quantal opening of the vagina were noted in immature mice and bilaterally ovariectomized rats (both dose groups for each). A dose-related decrease in the number of leukocytes and increases in cornified and epithelial cell counts were also reported for both species.

No significant changes in body weight were observed. The authors concluded that the ether-soluble portion of the methanol extract of *Ricinus communis* var. *minor* seeds possesses anti-implantation, anticonceptive, and estrogenic activity in rats and mice when administered subcutaneously (Okwuasaba *et al*., 1991).

Raji *et al.,* (2006) suggested that if the estrogenic effects of *Ricinus communis* seed extract can be exerted via the steriodogenic pathway as reported on its effect on female reproduction, *Ricinus communis* seed extract could also affect the biosynthesis and release of male androgens(which are themselves steroids) which may in turn impact negatively on male reproductive functions. Raji *et al.,* (2006) investigated the impact of *Ricinus communis* seed extract on male reproductive functions and found that *Ricinus communis* seed extract negatively impacted on male reproductive functions. Significant decrease was reported for weight of reproductive organs, sperm functions, and serum level of testosterone in *Ricinus communis* extract treated rats in a dose dependent manner. Raji *et al.,* (2006) concluded by suggesting that the observed adverse impact on male reproductive functions appear to be mediated via gonadal disruption in testosterone secretion.

2.35 Studies on outcomes in the use of *Ricinus communis oil* in labour induction:

Davis (1984) investigated the use of castor oil to stimulate labor using 196 patients with premature rupture of membranes (PROM), at least 4 h in duration, who were between 37 and 42 weeks of gestation. Patients were identified by reviewing charts of all patients who were admitted at an out-of-hospital birthing center from 1976 through 1981. Of the 196 patients, 107 (mean age = 28.6 years) were dosed orally with castor oil (2 oz) and 89 (mean age=27.6 years) were not. Castor oil was administered only to PROM patients who had a latency period of at least 4 h. All patients were observed for labor onset for 24 h after the onset of PROM. If labor did not occur at this time, the patients were transferred to a hospital for oxytocin stimulation. The longest interval between rupture of the membranes and delivery was 48 h. Of the 107 patients dosed with castor oil, 80 (75%) had labor onset. Spontaneous labor occurred in 52 (58%) of the

89 control patients. This difference between patients dosed with castor oil and controls was statistically significant ($p < .05$). The interval between castor oil administration and the onset of labor ranged from 1 to 13 h (mean = 4 h). Labor outcomes were also evaluated for type of delivery, incidence of oxytocin stimulation, and infant well-being. The need for cesarean sections was nearly three times greater in the control group (15.7% incidence) than in patients dosed with castor oil (5.6% incidence). This difference was found to be statistically significant ($p < .01$). Additionally, the group dosed with castor oil did not require oxytocin stimulation as frequently (36% incidence) when compared to the control group (43% incidence), and the difference was not statistically significant. Infant outcomes in the two groups were studied by observing Apgar scores at 1 and 5 min, and looking for any evidence of meconium staining. Two infants with Apgar scores of <7 were reported for both groups. At the time of membrane rupture, the presence of meconium was very low in both groups. Meconium staining was not observed after dosing with castor oil. It is also important to note that there were no maternal deaths and no significant maternal morbidity. The authors concluded that castor oil can be used safely and effectively to stimulate labor (Davis, 1984).

In a study by Mitri *et al*., (1987), the amniotic fluid was examined at the time of rupture of membranes in 478 of the 498 women in labor. The amniotic fluid was meconium-stained in 174 subjects (mean age = 28.5 ± 0.65 years) and clear in the remaining 304 subjects (mean age = 25.9 ± 0.37 years). Cesarean section, low Apgar score, and the recent ingestion of castor oil were significantly more common in subjects with meconium-stained amniotic fluid. The authors concluded that the passage of fetal meconium was predictive of poor labor outcome in terms of Apgar scores and the need for cesarean section. It was recommended that women who take

castor oil in late pregnancy should be identified as a high-risk group for the passage of fetal meconium.

In a case report by Steingrub *et al*., (1988), a 33-year-old pregnant female (at week 40 of gestation) ingested castor oil to induce labor. Within 60 min of ingestion, cardiopulmonary arrest occurred and was reportedly due to amniotic fluid embolism.

Garry *et al*., (2000) evaluated the use of castor oil to induce labor in 52 pregnant women (mean age = 24.8 ± 6.7 years) at Saint Mary's Hospital in Brooklyn, New York. The untreated control group consisted of 48 pregnant women (mean age=24.4 ± 4.9 years). The two groups of women did not differ in maternal age, parity, or gestational age. Castor oil was administered as a 60-ml dose in orange or apple juice, and its use was deemed successful only if active labor began within 24 h. Labor was defined as one or more contractions every 5 min, with cervical dilatation of 4 cm or more. Active labor was induced in 30 (57.7%) of the 52 women dosed with castor oil, compared to 2 of the 48 women in the control group ($p < .001$). The caesarean section rate for women dosed with castor oil was 19.2% (10 of 52women), compared to 8.3% (4 of 48 controls) in the untreated control group. No relationship between dosing with castor oil, birth weight, and mode of delivery ($p = .66$) was found. Additionally, no adverse outcomes for the mother or fetus were identified. The authors concluded that women dosed with castor oil had an increased likelihood of initiation of labor within 24 h, compared to women who were not dosed with castor oil.

2.36 *Ricinus communis* oil and uterine tissue contraction:

O'Sullivan *et al.*, (2009) reported that *Ricinus communis* oil used as vehicle for 17 hydroxyprogesterone caproate (17HP) exerted an uterotonic effect in human myometrium in pregnancy. Their study was based on the possibility that castor oil used as vehicle for 17HP,

exerts an independent effect on human uterine contractility. In the study potential effects on contractility of exposure of isolated human myometrial preparations, obtained during pregnancy, to castor oil was evaluated. Biopsies of human myometrium were obtained at elective cesarean section (n=8). Dissected myometrial strips suspended under isometric conditions, had contractility induced, for a 30 minute period using oxytocin (0.5nM). Strips were removed from the tissue bath and inserted in Castor Oil or physiological salt solution (PSS), for a 30 minute period. Strips were then re-suspended under isometric conditions and exposed to further oxytocin challenge. Contractile integrals were expressed in relation to the first challenge, and compared between study and control groups. Strips exposed to castor oil demonstrated increased contractile activity elicited by oxytocin after the castor oil exposure (165.53%± 17.03%, n=8, P=0.004), in comparison to that measured prior to its exposure. For strips exposed to PSS only, the second oxytocin challenge reduced contractile activity (72.57%±7.48%, n=8, P=0.003). Comparison of the contractile activity, between castor oil and PSS exposed strips, revealed a significant increase in contractile activity for those exposed to castor oil ($p<0.001$). O'Sullivan *et al.*, (2009) concluded from their finding that exposure of human myometrial preparations to castor oil resulted in an enhanced state of contractility in in-vitro model, in comparison to control experiments.

2.37 Provocative Tests on *Ricinus communis* oil:

Fujimoto *et al.*, (1997) conducted a study involving 332 patients (25 males, 307 females; ages ≈20 to 70) suspected of having cosmetic contact dermatitis. The subjects were patch-tested with numerous cosmetic products and cosmetic ingredients. Patch tests (Finn chambers) were applied to the back for 48 h, and the test sites were examined at 30 minutes after patch removal, 1 day

later, and 4 to 5 days after patch removal. None of the 49 patients patch-tested with castor oil had a positive reaction.

In a study by Hino *et al*., (2000), 346 patients (31 males, 315females; ages ≈ 20 to 70 years) suspected of having cosmetic dermatitis were patch tested with various cosmetic products and cosmetic ingredients. Patch tests (Finn chambers) were applied to the back for 48 h. Test sites were examined according to the schedule in the preceding study. Of the 76 patients patch-tested with castor oil, one had a positive reaction. This reaction was observed only during the second reading (i.e., the day after the 30-min reading).

2.38 Significance of anogenital distance (AGD)

Anogenital distance is a sexually dimorphic secondary sex characteristic in many mammalian species. Anogenital distance may be used to measure the degree of demasculinization of males as a consequence of developmental exposure to androgen receptor (AR) antagonists (e.g., vinclozolin, procymidone, flutamide, linuron, prochloraz, etc.), 5-alpha reductase inhibitors (finasteride), or compounds that inhibit steroidogenesis (some phthalates). Likewise, AGD is useful in measuring the degree of masculinization of females exposed during sexual differentiation to androgenic compounds such as testosterone or the anabolic growth stimulant trenbolone used in cattle. Anogenital distance and body weight are normally measured in rats at birth or 2 days of age. As the animal grows, increased activity and variability in growth rates increase the variability of this measurement. However, decreased male AGD and increased female AGD observed in adult rats at necropsy usually demonstrate that endocrine disrupting compounds do permanently alter AGD (Ostby and Gray, 2004).

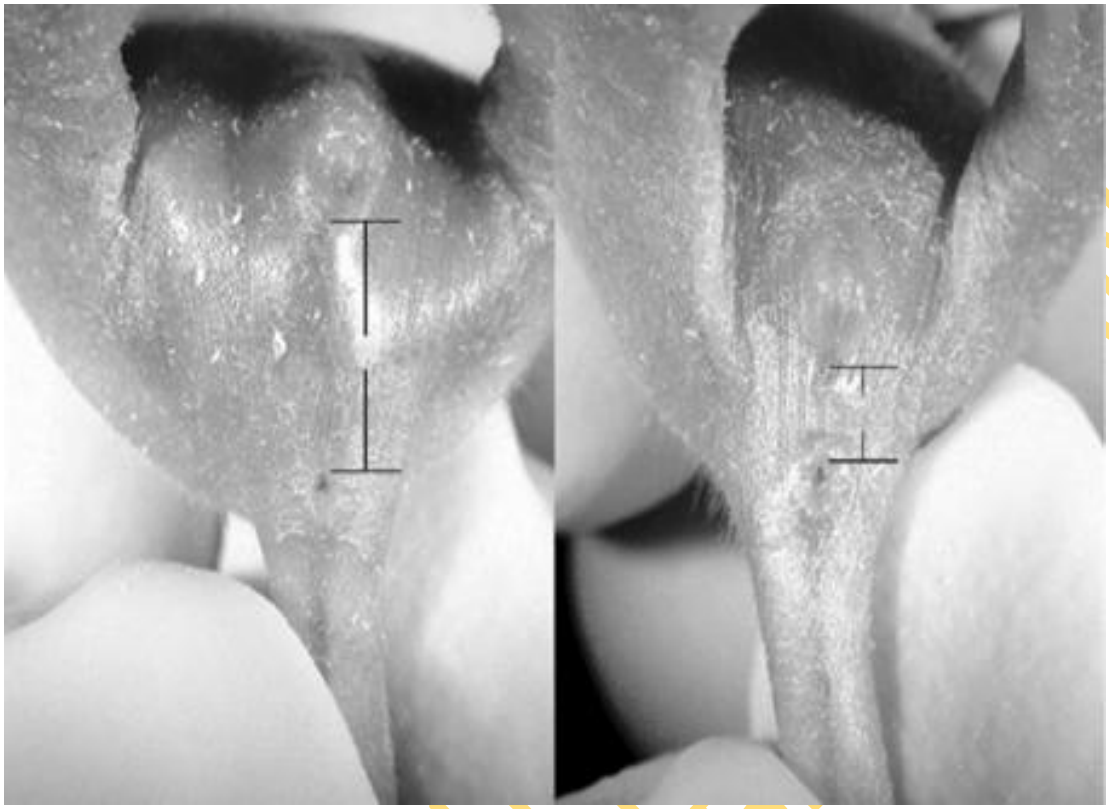
Anogenital distance is a reliable predictor of permanent alterations of the reproductive system that are not often apparent until the animal reaches sexual maturity. Similarly, increased AGD in

female pups is associated with decreased numbers of nipples (as infants and adults) and malformations of the reproductive tract.

2.39 Measurement of Anogenital distance:

At necropsy, AGD can be measured by placing the animal with the base of the tail on the edge of a table. The thumb is then used to secure the tail to the side of the table and place the index finger above the phallus to maximally stretch the skin in the perineal area. Then a vernier caliper is used to measure the distance between the posterior base of the phallus and the anterior rim of the anus. If pup body weight is significantly reduced by treatment, then adjustment is made for the AGD using body weight as a covariate in the statistical analysis.

Figure 2.1 Diagram showing male and female anogenital distance



Male **Female (**Ostby &Gray, 2004)

2.40 Detecting puberty—vaginal opening (VO) and preputial separation (PPS) in rats

The onset of puberty in the rat can be evaluated by recording the body weight and age of the rat at vaginal opening (VO) or preputial separation (PPS). Although direct exposure from weaning through puberty may be the optimum period of time to alter this endpoint, in utero/lactational exposure can also impact puberty and consequently should be checked in transgenerational studies.

Vaginal opening occurs when the rise in circulating ovarian hormones induces apoptosis of the vaginal membrane cells and development of the vaginal canal. The day of vaginal opening normally coincides with the day of first estrus and thus marks the initiation of the onset of estrous cyclicity in rats. The first few estrous cycles are typically longer and irregular after VO. Vaginal opening is useful to identify/evaluate EDCs that accelerate (estrogen) or delay (anti-estrogens, inhibitors of aromatase or inhibitors of hypothalamic-pituitary maturation) puberty in female rats (Ostby and Gray, 2004). On occasion, females display a transient thread of tissue along the midline of the vaginal opening, which persists for several days or occasionally is permanent. For this reason, the age and body weight at the onset of VO should be noted as well as the completion of VO, with dissolution of the thread, if present. Authors have found that female rats exposed in utero to Ah receptor agonists often display a permanent thread across the vaginal opening. Preputial separation in rats (complete manual retraction of the prepuce) occurs when increasing testicular androgen production produces cornification of the balano-preputial epithelial cells permitting the prepuce to retract along the length of the glans penis. The age at PPS is useful to identify antiandrogens which can delay puberty through altered development of the balano-preputial membrane (permanent incomplete PPS) or interfere with the normal rise in circulating androgens. Malformations such as incomplete PPS or hypospadias, which prevent

accurate PPS data collection, are reported as a malformation when the adults are necropsied. Occasionally, males display a transient thread of tissue along the frenulum of the glans, which persists for several days or, on rare occasions, is permanent. For this reason, the age and weight at the onset of PPS should be noted as well as the completion of PPS, with the dissolution of the thread, if present (Ostby and Gray, 2004). Also male rats with hypospadias may display a permanent thread on the glans or they are so malformed that PPS does not occur at all. If time does not permit examining a large number of rats daily for VO or PPS, then the rats can be examined two or three times per week and the age of VO or PPS and body weight at puberty recorded. These data can then be analyzed and reported as the percentage of females or males which opened or separated on a given day for each treatment group (Ostby and Gray, 2004)

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animals

Adult male and female rats (Wistar strain) obtained from the Central Animal House, Lagos State University College of Medicine Ikeja, were used for the experiments. Females were nulliparous and males used for mating were certified fertile by isolated mating techniques. Animals were allowed to acclimatize for three weeks to laboratory conditions, housed singly in cages, and fed with rats' cubes (Ladokun feeds limited Ibadan, Nigeria) and water for the entire duration of the study. A 12- hour dark-light period was maintained throughout the study. The weights of the rats were 180-200g and 200-250g for the female and male respectively. Rats were chosen for this study because they cost less, require small space, have short gestation periods and life span of generations. They also have large litter size and can also be easily standardized (Farris and Griffith, 1949).

3.2 Plant material:

Seeds of *Ricinus communis* plant were collected from Oyo town, Oyo State, South Western Nigeria between July/August 2008. The seeds were authenticated at the herbarium of the Forestry Research Institute of Nigeria, (FRIN), Ibadan. (Voucher no: 106878). The seeds were air dried before extraction.

3.3 Extraction, physicochemical analysis, gas chromatography and molecular spectroscopy of *Ricinus communis* (RC) seed

Seeds of *Ricinus communis* were air dried to a constant weight. Mortar and pestle were used to crush the beans into a paste (cake) in order to release castor fat for extraction. Pulverized seeds (1.5kg) were extracted with 5 litres of methanol by cold extraction. The pulverized seeds were soaked for 72 hours after which the mixture was filtered to remove the marc. The mixture separated into 3 layers tagged RC_A, RC_B and RC_C. RC_A was a golden coloured oily layer (368.9g), with a yield of 24.6%, RC_B was a brownish resinous substance (20g), and yield of 1.3%, while RC_C was a dusty brown substance (residue) (55g), and yield of 3.7%. The separated mixtures were evaporated of the solvent in a rotatory evaporator at 37^0C and stored at 0^0C prior to further analysis (Kathren *et al*., 1959).

RC_A was the major component of interest and was thus subjected to further physicochemical analysis.

3.4 Physicochemical screening of oily fraction RC_A of *Ricinus communis* seed:

Physicochemical analysis on the oil was done at the Nigeria Institute of Science and Technology, Samonda, Ibadan, Nigeria. Density, Saponification value, acid value, and free fatty acid value were all determined according to standard procedure detailed as follows.

3.5 Determination of saponification value

Indicator method was used as specified by ISO 3657, 1988, Nkpa *et al*., (1989). 2g of the sample was weighed into a conical flask; 25ml of 0.1N ethanolic potassium hydroxide was then added. The content which was constantly stirred was allowed to boil gently for 60min. A reflux

condenser was placed on the flask containing the mixture. Few drops of phenolphthalein indicator was added to the warm solution and then titrated with 0.5M HCl to the end point until the pink colour of the indicator just disappeared. The same procedure was used for the blank.

The expression for saponification value (S.V.) is given by: $S.V = 56.1N (V_0-V_1)/M$, where V_0 = the volume of the solution used for blank test; V_I = the volume of the solution used for determination; N = Actual normality of the HCl used; M = Mass of the sample.

3.6 Determination of acid value

25ml of diethyl ether and 25ml of ethanol was mixed in a 250ml beaker. The resulting mixture was added to 10g of oil in a 250ml conical flask and few drops of phenolphthalein were added to the mixture. The mixture was titrated with 0.1M NaOH to the end point with consistent shaking for which a dark pink colour was observed and the volume of 0.1M NaOH (V_0) was noted. Free Fatty Acid (FFA) was calculated as: $V_0/W_0 \cdot 2.82 \cdot 100$, where 100ml of 0.lM NaOH = 2.83g of Oleic acid, W_0 = sample weight; then Acid Value = FFA·2.

3.7 Determination of Specific gravity value

Density bottle was used to determining the density of the oil. A clean and dry bottle of 25ml capacity was weighed (W_0) and then filled with the oil, stopper inserted and reweighed to give (W_1). The oil was substituted with water after washing and drying the bottle and weighed to give (W_2). The expression for specific gravity (Sp.gr) is: $Sp.gr = (W_1-W_0)/(W_2-W_0)$ = Mass of the substance / Mass of an equal volume of water.

3.8 Determination of Gas chromatography and molecular spectroscopy

Gas chromatography and molecular spectroscopy (GCMS) analysis of the oil was done using an Agilent Technologies 6890GC interfaced to an Agilent 5973N mass selective detector. HP-5MS

column with diameter of 30X0.25mmX1.0µm was used with helium as carrier gas at a flow rate of 22cm/sec. The gas chromatography oven temperature was initially 50^0C held for 5mins at 2^0C/min then 250^0C held for 3mins. The injector temperature was at 250^0 C with a split ratio of 1:30 and MS detector at 280^0 C. Percentage compositions were then obtained from electronic integration measurement using flame ionization detector at 280^0 C. The peak numbers and relative abundance of the chemical components with their retention time were then determined.

3.9 Acute oral toxicity test:

Sequential limit test of the Organization for Economic Cooperation and Development (OECD, 2001) protocol was utilized for this study. Male and female rats were tested at both 2000mg/kg and 5000mg/kg. The Limit Test is a sequential test that uses a maximum of 5 animals at test doses of 2000, and 5000 mg/kg. The procedures for testing at 2000 and 5000 mg/kg are slightly different as explained below.

3.10 Description of the sequential limit test (OECD GUIDELINE 425)

3.11 Selection of animal species

The preferred rodent species is the rat although other rodent species may be used. Normally female rats are used. This is because literature surveys of conventional LD50 tests show that usually there is little difference in sensitivity between sexes. In those cases where differences are observed, females are generally slightly more sensitive. However, if knowledge of the toxicological or toxicokinetic properties of structurally related chemicals indicate that males are likely to be more sensitive then this sex should be used. When the test is conducted in males, adequate justification should be provided. Healthy young adult animals of commonly used

laboratory strains should be employed. Females should be nulliparous and non-pregnant. At the commencement of its dosing, each animal should be between 8 and 12 weeks old and its weight should fall in an interval within ± 20 % of the mean initial weight of any previously dosed animals.

3.12 Housing and feeding conditions

The temperature in the experimental animal room should be 22°C (± 3°C). Although the relative humidity should be at least 30 % and preferably not exceed 70 % other than during room cleaning the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light and 12 hours dark. The animals are housed individually. For feeding, conventional rodent laboratory diets were used with an unlimited supply of drinking water.

3.13 Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions. As with other sequential test designs, care was taken to ensure that animals are available in the appropriate size and age range for the entire study.

3.14 Preparation of doses

In general test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture is to be tested, however, the use of the undiluted test substance, i.e., at a constant concentration, may be more relevant to the subsequent risk assessment of that substance. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1 mL/100g of body weight; however

in the case of aqueous solutions, 2 mL/100g body weight can be considered. With respect to the formulation of the dosing preparations, the use of an aqueous solution/suspension/emulsion is recommended wherever possible, followed in order of preference by a solution/suspension/emulsion in oil (e.g. corn oil) and then possibly solution in other vehicles. For vehicles other than water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

3.15 Administration of doses

The test substance is administered in a single dose by gavage using a stomach tube or a suitable intubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a period not exceeding 24 hours. Animals should be fasted prior to dosing (e.g., with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3-4 hours). Following the period of fasting, the animals should be weighed and the test substance administered. The fasted body weight of each animal is determined and the dose is calculated according to the body weight. After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

3.16 Limit test at 2000 mg/kg

One animal is dosed at the test dose, if the animal dies; the main test is conducted to determine the LD_{50}. If the animal survives, four additional animals are dosed sequentially so that a total of five animals are tested. However, if three animals die, the limit test is terminated and the main test is performed. The LD_{50} is greater than 2000 mg/kg if three or more animals survive. If an

animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The results are evaluated as follows (√=survival, X=death).

3.17 Decision rule for limit test at 2000mg/kg BW:

Test five animals, the LD_{50} is less than the test dose (2000 mg/kg) when three or more animals die, the LD_{50} is greater than the test dose (2000 mg/kg) when three or more animals survive.

3.18 Limit Test at 5000 mg/kg BW

Exceptionally, and only when justified by specific regulatory needs, the use of a dose at 5000 mg/kg may be considered. For reasons of animal welfare concern, testing of animals in GHS Category 5 ranges (2000-5000mg/kg) is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment.

One animal is given the test dose, if the animal dies; the main test is conducted to determine the LD_{50}. If the animal survives, two additional animals are dosed. If both animals survive, the LD_{50} is greater than the limit dose and the test is terminated (i.e. carried to full 14-day observation without dosing of further animals). If one or both animals die, then dose an additional two animals, one at a time. If an animal unexpectedly dies late in the study, and there are other survivors, it is appropriate to stop dosing and observe all animals to see if other animals will also die during a similar observation period. Late deaths should be counted the same as other deaths. The results are evaluated as follows (O=survival, X=death, and U=Unnecessary).

3.19 Decision rule at 5000mg/kg BW

The LD50 is less than the test dose (5000 mg/kg) when three or more animals die. The LD_{50} is greater than the test dose (5000 mg/kg) when three or more animals survive.

3.20 Experimental design

Two study protocols were undertaken; the first study protocol explored the effects of R.C oil at different gestation periods on haematological, hormonal, biochemical & histopathology of pregnant rats, while the second study protocol target specifically litter from female rats dosed at different gestation periods.

3.21 Experimental protocol 1:

For the determination of haematological, hormonal, histopathology of reproductive organs and biochemical indices in pregnant Wistar rats treated with *Ricinus communis* oil at different gestation periods.

Twenty-five mature nulliparous female albino rats (10weeks) with normal estrous cycle were used. Male rats for mating were certified fertile by isolated mating technique. Mating was confirmed by the presence of a sperm positive vaginal smear or copulatory plug (Stump *et al.*, 2007). Day after which either of this was found was taken as gestational day 1((Foster *et al*., 2002). Pregnant rats were then randomly assigned to treatment groups in a manner that provided for comparable body weight, means and distribution across groups. There were five animals per group and dosage for all groups was 950mg/kg bwt, which is the recommended therapeutic dose in adult humans (Drugstore.com, Inc.2004) via oral dosing syringe except group 1 (control) which received distilled water.

Group 1: Control animals received distilled water,

Group 2: Were administered *Ricinus communis* oil between gestation days (GD) 1-7,

Group 3: Were administered *Ricinus communis* oil between GD 7-14,

Group 4: Were administered *Ricinus communis* oil between GD 14-21,

Group 5: Were administered *Ricinus communis* oil between GD 1-21.

These treatment groups were chosen in order to target all the critical period of intrauterine life (i.e. early, mid, late and entire gestation periods). Blood samples were collected via the orbital sinus for haematological, hormonal and biochemical indices. Weights of the animals were also taken once every other day. The animals were subsequently sacrificed on GD 21. The ovary and uterus were also taken for histopathological screening.

3.22 Experimental protocol 2:

Twenty-five mature nulliparous female albino rats (10weeks) with normal estrous cycle were used. Male animals for mating were certified fertile by isolated mating technique. Mating was confirmed by the presence of a sperm positive vaginal smear or copulatory plug. Day after which either was found was taken as gestational day 1. Pregnant rats were randomly assigned to treatment groups in a manner that provided for comparable body weight, means and distribution across groups. There were five animals per group and dosage for all groups except control was 950mg/kg bwt via oral dosing syringe.

Group 1: Control animals received distilled water,

Group 2: Were administered *Ricinus communis* oil between gestation days (GD) 1-7,

Group 3: Were administered *Ricinus communis* oil between GD 7-14,

Group 4: Were administered *Ricinus communis* oil between GD 14-21,

Group 5: Were administered *Ricinus communis* oil between GD 1-21. The animals were allowed to deliver sequel to which the following parameters were determined in the mother and litter.

1. Gestational length in all the groups
2. Weight of pregnant rats throughout gestation period
3. litter size
4. Weekly pups weight until weaning at postnatal day (PND) 21. After weaning pups were separated according to sex.
5. Morphometric data (body length, abdominal diameter and head diameter) at postnatal day 1 and at sacrifice (PND90)
6. Anogenital distance and index of male and female pups (using digital calipers) distance between anus and genital tubercle on postnatal day 1(PND 1), and at PND 90.
7. Weight and day on attainment of puberty (i.e. period of balanopreputial separation, separation of prepuce from glans in male and vaginal patency of female. The vagina was considered to be patent when no membranous connections were observed on physical examination in litters of individual groups. (This commenced at PND 25 & 35 for female & male rats respectively).
8. Evaluation of sperm parameters in F_1 male after attainment of puberty(at sacrifice PND 90)
9. Absolute and relative organ weights i.e. pituitary gland, testis, epididymis, seminal vesicle, prostate, ovary, uterus

10. Histopathology of testis, epididymis, ovary and uterus of F1 offspring in all the groups
11. Fertility test in F1 male and female at maturity
12. **Mating index**: determined as no of males or females mating/no of males or females cohabited X100
13. **Fertility index**: determined as no of cohabited female becoming pregnant/no of non pregnant couple cohabited X100
14. **Gestation Index**: determined as no of females delivering live young/no of females with evidence of pregnancy X100
15. **Live Birth Index**: determined as no of live offspring/no of offspring delivered X100
16. **Day survival index (viability index):** determined as no of life offspring at lactation day4/no of life offspring delivered X100
17. **Lactation index/weaning index: determined as:** no of live offspring at day 21/no of life offspring bornX100.When no standardization is done it is weaning index but when standardization is done it is lactation index.

Items 11-17 were determined according to United States Environmental Protection Agency (EPA) guideline on generational reproductive toxicity risks assessment. (U.S. EPA, 1996)

3.23 Morphometric data determination:

Head diameter, abdominal diameter, and body length were measured using a vernier caliper. Animals were gently held firmly to allow for ease in measurement (Ostby and Gray, 2004).

3.24 Mating experiment on F_1 male and female

Isolated mating technique as described by the EPA guideline was employed. Randomly selected male from each of the treated groups were paired with randomly selected females from each

treated groups at ratio 1:1 for 14days. Formation of copulatory plug was taken as evidence of positive mating. Mating and fertility indices were then determined. Pregnant female rats were allowed to deliver. Morphometric, live birth, day survival and lactation indices were then determined in F_2 male and female offspring.

Treated male F_1 rats were also paired with untreated female rats at ratio 1:1 for 14 days. Positive mating was determined as described above and pregnant female rats were allowed to also deliver and data collected as described above (Section 3.24).

3.25 Measurement of anogenital distance (AGD) and index

Anogenital distance was measured by placing the animal with the base of the tail on the edge of a table. The thumb was then used to secure the tail to the side of the table and the index finger placed above the phallus to maximally stretch the skin in the perineal area. Then, a vernier caliper was used to measure the distance between the posterior base of the phallus and the anterior rim of the anus. The anogenital index was measured by adjusting the AGD with body weight, (i.e. anogenital distance divided by cube root of body weight) (Reynolds *et al*., 1999).

Anogenital distance is a sexually dimorphic secondary sex characteristic in many mammalian species and can be used to measure the degree of demasculinization of males as a consequence of developmental exposure to androgen receptor (AR) antagonists, 5-alpha reductase inhibitors, or compounds that inhibit steroidogenesis (Fisher, 2004). Likewise, AGD is useful in measuring the degree of masculinization of females exposed during sexual differentiation to androgenic compounds or anabolic growth stimulant. Increased activity and variability in growth rates increase the variability of this measurement. However, decreased male AGD and increased female AGD observed in adult rats at necropsy demonstrate that endocrine disrupting

compounds do permanently alter AGD. Anogenital distance is a reliable predictor of permanent alterations of the reproductive system that are not often apparent until the animal reaches sexual maturity. Similarly, increased AGD in female pups is associated with decreased numbers of nipples (as infants and adults) and malformations of the reproductive tract (Fisher, 2004).

3.26 Determination of puberty (vagina opening in female and preputial separation in male)

3.26a. Vaginal opening:

Female rats were checked daily for vaginal opening (VO) starting from PND 25 to ensure the day of VO was detected in all rats (Guzman *et al*. 2006). Monitoring of each rat was done daily until the process is complete. The index finger was occasionally used to gently rub across the vaginal area to determine if the vaginal membrane was present or if the layers of dying cells are simply obscuring the vaginal opening. When VO was initiated and completed for each rat, the mean ages and body weights of offspring for each treatment groups were calculated and then checked for statistical significance. The vagina was considered to be patent when no membranous connections were observed on physical examination in female litter of individual groups starting from PND 25 (Ostby and Gray, 2004).

3.26b Preputial separation (PPS)

Preputial separation in rats (complete manual retraction of the prepuce) occurs when increasing testicular androgen production produces cornification of the balano-preputial epithelial cells permitting the prepuce to retract along the length of the glans penis.

Male rats were checked daily beginning at 35th day of age to ensure no rats have preputial separation before being checked (EPA, 1996). Gentle pressure was applied to the prepuce to retract the prepuce and expose the glans penis. Preputial separation was completed when the entire perimeter of the prepuce can be retracted evenly around the base of the glans penis. When

PPS was completed, the mean age, body weight, and standard errors by treatment groups were checked for statistical significance.

3.27 Collection of serum from pregnant rats:

Pregnant rats were bled from the orbital sinus at gestation day 20. Blood (2ml) was then collected into polyethylene tubes and allowed to clot at 4^0C for 1hour. The blood samples were then centrifuged at 3500g for 15 minutes at 4^0C. Serum samples were then kept at -10^0C until assayed for biochemical and hormonal parameters. For heamatological studies, blood samples (2ml) were collected in heparinized bottles. Animals were subsequently sacrificed via cervical dislocation.

3.28 Collection of serum from rats at puberty:

Rats were bled from the orbital sinus and blood samples (2ml) were then collected into polythene tubes and allowed to clot for 1 hour. Blood samples were then centrifuged at 3500g for 15 minutes at 4^0C. Serum was then aspirated and stored until assayed for hormonal parameters.

3.29 Determination of serum alanine amino transferase (ALT):

Serum alanine aminotransferase and aspartate aminotransferase were assayed according to the methods of Mohun and Cook (1957) and Reithman and Frankel (1957).

L- alanine: 2-oxoglutarate amino transferase catalyses the reaction, L- alanine + 2-oxoglutarate=L-glutamate+pyruvate. Pyruvate reacts with 2, 4-dinitrophenyl hydrazine to produce hydrozone on the addition of sodium hydroxide. The optical density of the colour developed was then measured at 505nm wavelength.

3.30. Determination of serum aspartate aminotransferase (AST):

L- aspartate : 2- oxoglutarate aminotransfarase catalyses the reaction, L- aspartate + 2-oxoglutarate= L-glutamate+ oxaloacetate. The unstable oxaloacetate was then quantitatively decarboxylated to pyruvate with 2, 4- dinitrophenylhydrazine produced an intensely coloured hydrazone on the addition of 0.4N sodium hydroxide solution. Measurement of the optical density at 505nm then provided a measure of the enzyme activity.

3.31 Procedure for determination of ALT:

Three tubes were set up, each of the three test tubes contain 1ml of ALT-buffered substrate in a water bath at 37^0C. 0.2ml of a sample was introduced into two of the three test tubes and allowed to stay for 30minutes. After 30 minutes, 1ml of 0.001M 2,4-dinitrophenyl hydrazine was added to two of the test tubes on the water bath and 0.0ml of the sample added to the third test tube. Contents of the three test tubes were then mixed thoroughly and allowed to remain in the water bath for another 20mins. Thereafter 10ml of 0.4M sodium hydroxide was added to the three test tubes, mixed properly and allowed to remain in the water bath for another 10mins fro dinitrophenyl-hydrazine colour to develop.

The optical density of the solution was then read against a water blank at 505nm in a SP600 Spectrophotometer (SP 6100 model, Jenway, England).

The same procedure was employed for AST except that the reaction was allowed to proceed for 60mins. In addition two drops of aniline citrate were added to the reaction medium before 2, 4-dinitrophenylhydrazine reagent was added.

Calibration curves were prepared by using different concentration of pyruvate and carrying the standard through the same procedures as the serum sample.

3.32 Determination of Lipid and lipoprotein

Total cholesterol, triglyceride, low density lipoprotein cholesterol and high density lipoprotein cholesterol levels were determined by enzymatic colourimetric method. The determination was based on the formation of colour after enzymatic hydrolysis and oxidation. The indicator quinoneimine used was formed from H_20_2 and 4-amino-antipyrine in presence of phenol. The assay kit was obtained from Randox Laboratory Ltd (Co Atrim UK). Contents of the kit were standard cholesterol (5.17mM), phosphate buffer (0.1M), chloro-4-phenol (Chromogen; 5.0mM), enzymes, cholesterol oxidase (100.0 IU/L), cholesterol esterase (170 IU/L), peroxidase (1200 IU/L), cholic acid sodium salt (2.0mM) and 4-amino-antipyrine (0.3mM). Equal volumes of the enzymes and buffer were mixed gently after standing for 5-10min serving as a working reagent. 10µl of each sample was added to 1.0ml of the working reagent. 1.0ml of the working reagent was taken as a blank. The standard solution was prepared by mixing 10µL of the standard with 1.0ml of the working reagent. The preparation was mixed and incubated for 5min at 37^0C. The spectrophotometer (Boehringer Mannheim) was set at zero with the blank while the absorbance of the sample (A_{SAMPLE}) and standard (A_{STD}) were read against the blank at 520nm.

Concentration (mM) = (A_{SAMPLE} /A_{STD}) x standard concentration.

LDL-Cholesterol is estimated according to Friedewald's formula (Friedewald *et al.*, 1972).

3.33 Determination of haematological parameters:

3.33a Packed cell volume (PCV):

The PCV was determined using the micro haematocrit method as described by Jain (1986). Blood samples were drawn into plain capillary tubes by capillary traction. The capillary tubes

were then placed in a microhaematocrit centrifuge with the sealed end pointing outward and then centrifuged at 3000g. PCV were then read from a micro haemtocrit reader.

3.33b Determination of haemoglobin concentration:

This was estimated using the cyanomethaemoglobin method as described by Jain (1986). 4mls of Drabkins solution(prepared with 1gm of NaHC03,0.05g KCN and 0.02gm K3Fe(CN)6, all mixed in 500mls of distilled water) was carefully transferred into a clean teest tube. 0.02ml of blood sample was then transferred into the test tube to give a blood to reagent dilution of 1.200. The mixture was left for 10min in order to allow for cyanomethaemoglobin to form. Optical density was then read in a spectrophotometer at a wavelength of 540nm, using 4ml of Drabkins solution as blank. Optical density (OD) of a haemoglobin standard of known concentration was then determined and the value used to calculate the haemoglobin concentration of the sample thus: Hb conc. (g/dl of blood) = O.D. of blood X Hb. Conc of standard/O.D. of standard.

3.33c Determination of red blood cell counts:

Red blood cell count was done using haemocytomter and hagens solution (0.5gm mercury chloride, 1gm sodium chloride, 5g sodium sulphate and 200ml distilled water as diluents). Number of erythrocytes in 5 of the 25 squares in the central area of each chamber of the Neubauer haemocytometer was counted, i.e. 4 corners squares and the central one. Total number of erythrocytes obtained was then multiplied by depth (X10), area (X5) and dilution factor (X200). Hence for every E erythrocyte counted, the number of erythrocytes per milliliter in the original sample would be 10,000E.

3.33d Determination of total and differential leucocyte (WBC) values

White blood cell counts (WBC) were also made in a haemocytometer using white blood cell diluting fluid. Sum of WBC counts from the 4 large corner squares of the haemocytometer

chamber was multiplied by the depth (X10) and dilution factor (X20) and then divided by the squares counted (4). The number of WBC per milliliter in the original sample was 50X where X was the number of leucocytes counted in the 4 squares.

Differential WBC counts in which 100 cells per slide were counted were then made from Geimsa stained blood smear.

3.34 Sperm analysis:

Semen samples were collected from the cauda epididymis on pre- warmed microscope glass slide (27^0 C). Sperm analysis was done as described by Zemjanis (1970).

3.35 Epididymal progressive sperm motility

This was done immediately after the collection of the semen. Two drops of semen were placed on the microscope slide with two drops of warm 2.9% sodium citrate. It was then covered with a slip and examined under the microscope using X40 objective with reduced light. Sperm motility was then determined as a percentage.

3.36 Epididymal sperm viability:

Viability study (percentage of live spermatozoa) was done using the eosin/nigrosin stain. Semen was squeezed onto a microscope slide and two drops of the stain was added. The motile (live) sperm cells were stained. The stained and the unstained sperm cells were counted using X40 objectives of the microscope and an average for each was taken from which percentage viability was calculated.

3.37 Epididymal sperm morphology:

Sperm morphology was done by staining the sperm smears on the microscope slides with two drops of Walls and Ewas stain and then air-dried. The slides were examined under the

microscope using ×100 objectives under immersion oil. Morphological aberrations of spermatozoa were determined from total count of about 400 spermatozoa.

3.38 Epididymal sperm volume:

The epididymis was immersed in 5 mL normal saline in a measuring cylinder and the volume of fluid displaced was taken as the volume of the epididymis

3.39 Epididymal sperm count: Sperm count was done under a microscope with the aid of the improved Neubauer hemocytometer. Count was done in five large Thoma square and adjustment was made for volume of the normal saline added. The result was expressed as count $X10^{6}$ $ml^{-1.}$

3.40 Organ collection:

The animals were sacrificed by cervical dislocation and dissected (from the abdominal cavity) to collect organ of interest; testes, epididymis, seminal vesicle, prostate gland, pituitary gland, liver, ovary and uterus. The organs were cleared of adherent tissues, fats and then weighed immediately with an electronic weighing balance, model DT 300 with a capacity of 0.10-300g.

3.41 Histopathology of the testis, epididymis, ovary and uterus:

After weighing the organs were quickly fixed in Bouins fluid for about 20 hours. The tissues were then dehydrated by passing them through ethanol (70% ethanol for 2hrs; 95% ethanol for 2hrs, 100% ethanol for 2hrs, 100% ethanol for 2hrs and finally 100% ethanol for 2hrs). This gradual procedure was undertaken so as to remove the inherent water content of the organ in a gradual way considering osmotic dynamics.

The tissues were then cleared of the ethanol that the tissues had been bathed in and to initiate and complete the process that would make cells transparent at microscopic level. The clearing was done with xylene for 6hours. The tissues were then infiltrated by placing them in molten paraffin

wax which served as support to the tissues for subsequent stage of sectioning. The tissues were infiltrated for 6hrs, thereafter the tissues were embedded; which was the positioning of the processed, infiltrated tissues in molten paraffin wax within an enclosure called mould. The embedded tissues were left until the wax solidifies. The tissues were then cut into blocks (little chunks) and they were held in position by paraffin wax. The blocks were then clamped and positioned for sectioning.

Sectioning was done with a microtome which cut only a thin slice of the original tissues at a preset thickness of 4µm. The satisfactory sections were picked up with microscope glass slides that had been coated on one side with glycerin egg albumin. The slides carrying the sections were then labeled with a diamond pencil, and arranged in a slide carrier and then put in an oven to dry.

The slides were then stained with haemotoxyline and eosin stain and thereafter dewaxed with xylene three times for 3mins each and then hydrated in 100% ethanol for 3mins. This was followed with hydration in 96% ethanol for 3mins, 75% ethanol for 3mins, and then water for 3mins. The slides were stained in haematoxyline for 15mins and the excess stain was then washed off. The slides were placed in 1% acid for 5 seconds, which was then washed off. The slides were thereafter counterstained in eosin for 3secs and then dehydrated in serial ethanol solutions (50%, 70%, 95%, and 100%). Slides that have been stained were cleared in xylene before they were mounted on the microscope for histological examination. Photomicrographs of the slides were then taken at 200 and 400 magnifications.

3.42 Assay of Testosterone, Luteinizing Hormone, Follicle Stimulating Hormone, Estrogen and Progesterone

3.43 Determination of Testosterone

Principle of assay: Testosterone (antigen) in the sample competes with horseradish peroxidase testosterone (enzyme-labeled antigen) for binding onto the limited number of anti-testosterone (antibody) sites on the microplates (solid phase).

After incubation the bound/free separation is performed by a simple solid-phase washing. The substance solution (H_2O_2 + TMB) is added. After an appropriate time has elapsed for maximum colour development, the enzyme reaction was stopped and absorbances were determined. Testosterone concentration in the sample is calculated based on a series of standards. The colour intensity is inversely proportional to the testosterone concentration of the sample.

3.44 Reagents, Materials supplied in Kit with Instrumentation

Intelco ELISA Kit for in vitro determination of testosterone was used for this assay.

Calibrator (C_0-C_1-C_2-C_3-C_4-C_5): 1.0ml. Before use, it was mixed for 5mins with rotating mixer. The standards have the following concentration of Testosterone.

	C_0	C_1	C_2	C_3	C_4	C_5
Ng/ml	0	5	10	25	50	100

Enzyme Conjugate: 12.ml. Testosterone-hrp conjugate

Microwell plate: 8x12 strips Anti-Testosterone IgG absorbed on microplates

Substrate Solution: 12ml H_2O_2-TMB 0.25g/l

Stop Solution: 12ml 0.15mol/l sulphuric acid

Distilled water: it was used but it was not part of the kit.

Disposable glass tubes

Automatic dispenser

Microplate reader

3.45 Procedure:

Assay was done in duplicate; two wells were prepared for each of the five points of the calibrator curve and each sample and one for blank.

The wells were then mixed thoroughly and then incubated at 37^{o} C for 1 hour. Thereafter the content of each well was removed and washed with 300µl of distilled water. The washing procedure was repeated by draining the water completely.

100µl of substrate solution was then added separately to the 3 wells containing calibrator, sample and blank. The wells were then incubated at room temperature (28-30^{o}C) for 15minutes in the dark. Thereafter 100µl of stop solution was added separately to the 3 wells containing the calibrator, sample and blank. The absorbance was read at 450nm against blank.

3.46 Luteinizing Hormone

Luteinizing hormone enzyme immunoassay (EIA) Kit was used for this assay.

3.47 Principle of assay:

The assay (LH enzyme immunoassay) is an immunometric ("sandwich") design, utilizing two anti-LH monoclonal antibodies. The first is directed against the alpha-chain of the molecule and

is attached to a magnetic particle. The second is directed against the beta-chain, and is labeled with alkaline phosphatase. The assay has three main stages:

(a) Immunoextraction: sample is incubated with magnetic anti-LH for 15 minutes at 37^{o}C. LH in the sample binds to the magnetic particles. Other serum components are removed by a magnetic wash step.

(b) Labelled Antibody Reaction: The magnetic particles are incubated with alkaline phosphatase labeled anti-LH for 1hour at 37^{o}C. The labeled antibody reacts with any bound to the magnetic particles after immunoextraction. Excess labeled antibody is removed by two magnetic wash steps.

(c) Colour Development: The magnetic particles were incubated with a coloured enzyme substrate for 30minutes at 37^{o}C. the presence of alkaline phosphatase caused a colour change from yellow to pink, the intensity of which is a measure of the amount of alkaline phosphatase, labeled antibody (and hence LH) bound to the magnetic particle. The reaction was terminated by addition of stop buffer and the optical density of each tube was measured. The LH concentration of test samples was interpolated from a calibration curve.

3.48 Reagent and Material Supplied in the kit

Luteinizing Hormone enzyme immunoassay (EIA) Kit (Immunometrics (UK) Ltd, London) was used for the assay.

Luteinizing LH EIA standards: purified human LH is provided lyophilized in horse serum. Kit's standards are calibrated against WHO IRP 80/552

LH EIA Enzyme Labelled Antibody: Provided in liquid form as 2.5ml of a 13.5 times concentrate.

LH EIA Magnetic Antibody: Provided as a 10.5ml suspension.

EIA Substrate Reagent: Provided in 1 glass bottle containing 400mg Phenolphthalein monophosphate.

EIA Substrate Buffer: Provided as 55ml ready to use.

LH EIA Assay Buffer (5x conc): Provided as 10ml of a 5 times concentrate of 0.05 M phosphate buffer pH 7.4 containing magnesium, sodium and zinc chlorides, bovine and murine serum proteins, a surfactant and 0.1% sodium azide.

LH EIA Wash Buffer (5 x conc): Provided as 35ml of a 5 times concentrate of 1.0 M

TRIS /HCL buffer, containing magnesium and zinc chlorides, a surfactant and 0.05% sodium azide.

EIA Stop Buffer: Provided as 120ml ready to use. Glycerine buffer pH 10.4 containing sodium hydroxide and a chelating agent.

LH EIA Internal OC Sample: Provided as 1ml lyophilized serum.

3.49 Procedure

Step 1: Immunoextraction of LH

100µl of sample was pippetted into tubes 1-100. Working suspension of LH EIA magnetic antibody (100µl) was also added to tubes 1-100. The tubes were covered and briefly vortex mixed. The tubes were then incubated in a water bath at 37^{o}C for 15 minutes. The tubes were then removed from water bath. 500µl of diluted LH EIA wash buffer was added to tubes 1-100

and it was vortex mixed. The rack of the tubes was placed onto a magnetic base. The tubes were kept on the magnetic separator for 10minutes. The supernatant liquid was then decanted from all the tubes.

Step 2: Labelled antibody reaction: 250µl diluted LH EIA labeled antibody was added to tubes 1-100. The tubes were covered and were briefly vortex mixed. The tubes were then incubated in a water bath at 37^{o}C for 1 hour. The rack of tubes was thereafter removed from the water bath. The tubes were then washed twice. The washing was done by adding 500µl of diluted LH EIA wash buffer was added to tubes 1-100 and vortex mixed. The rack of tubes was placed onto a magnetic base. The tubes were kept on the magnetic separator for 10minutes. The supernatant liquid was then decanted from all the tubes.

STEP 3: Colour Development Step: 500µl of substate solution was added to tubes 1-100. The tubes were covered and were briefly vortex mixed. The tubes were then transferred to a 37^{o}C water bath and were incubated for 30 minutes. After removing the tubes from the water bath, 1ml of EIA stop buffer was then added to tubes 1-100 and briefly vortex mixed. The rack was then placed onto a magnetic base for 10 minutes. The spectrophotometer was set at wavelength of 450nm. It was zero by using the substrate blank solution (Tube 101). The OD at 450nm of solutions in tubes 1-100 were then measured.

3.50 Follicle Stimulating Hormone

Principle of assay

The assay (FSH enzyme immunoassay) is an immunometric (“sandwich”) design, utilizing two anti-FAS monoclonal antibodies. The first is directed against the alpha-chain of the molecule and

is attached to a magnetic particle. The second is directed against beta-chain, and is labelled with alkaline phosphatase. The assay has three main stages like that of Luteinizing hormone:

(a) Immunoextraction: Sample is incubated with magnetic anti-FSH for 15 minutes at 37^{o}C. FSH in the sample binds to the magnetic particles. Other serum components are removed by a magnetic wash step.

(b) Labelled Antibody Reaction: The magnetic particles are incubated with alkaline phosphatase labeled anti-FSH for 1 hour at 37^{o}C. The labeled antibody reacts with any bound to the magnetic particles after immunoextraction. Excess labeled antibody is removed by two magnetic wash steps.

(c) Colour Development: The magnetic particle are incubated with a coloured enzyme substrate for 30 minutes at 37^{o}C. The presence of alkaline phosphatase caused a colour change from yellow to pink, the intensity of which is a measure of the amount of alkaline phosphatase, labeled antibody (and hence FSH) bound to the magnetic particle. The reaction was terminated by addition of stop buffer and the optical density of all tube is measured. The FSH concentration of test samples was interpolated from a calibration curve.

3.51 Reagent and Material Supplied in the Kit

Follicle Stimulating Hormone enzymeimmunoassay (EIA) Kit (Immunocentric (UK) Ltd, London) was used for this assay.

FSH EIA standards: Purified human FSH is provided lyophilized in horse serum. Kit's standards are calibrated against WHO IRP 70/549

FSH EIA Enzyme Antibody: Provided in liquid form as 2.5ml of a 13.5 times concentrate.

FSH EIA Magnetic Antibody: Provided as a 10.5ml suspension.

EIA Substrate Reagent: Provided in 1 glass bottle containing 400mg phenolphthalein monophosphate.

EIA Substrate Buffer: Provided as 55ml ready to use.

FSH EIA Assay Buffer (5x conc): Provided as 10ml of a 5 times concentrate of 0.05 M phosphate buffer, pH 7.4, containing magnesium, sodium and zinc chlorides, bovine and marine serum proteins, a surfactant and 0.5% sodium azide.

FSH EIA Wash Buffer (5x conc.):Provided as 35ml of a 5 times concentrate of 1.0M TRIS/HCL buffer, containing magnesium and zinc chlorides, a surfactant and 0.05% sodium azide.

EIA Stop Buffer: Provided as 120ml ready to use. Glycerine buffer pH 10.4 containing sodium hydroxide and a chelating agent

FSH EIA OC Sample: Provided as 1 ml lyophilized horse serum.

3.52 Procedure

Step 1: Immunoextraction of FSH

100µl of sample was pippeted into tubes 1-100. Working suspension of FSH EIA magnetic antibody (100µl) was added to tubes 1-100. The tubes were covered and briefly vortex mixed. The tubes were then incubated in a water bath at 37^{o}C for 15 minutes.

500µl of diluted FSH EIA was buffer was added to tubes 1-100 and vortex mixed. The rack of tubes was placed on the magnetic separator for 10minutes. The supernantant liquid was then decanted from all the tubes.

Step 2: Labelled antibody Reaction: 250µl diluted FSH EIA labeled antibody was added to tubes 1-100. The tubes were covered and briefly vortex mixed. The tubes were then incubated in a water bath at 37^{o}C for 1 hour. The tubes were then washed twice. The washing was done by adding 500µl of diluted FSH EIA wash buffer was added to tubes 1-100 and vortex mixed. The rack of the tubes was placed on the magnetic separator for 10minutes. The supernatant liquid was then decanted from all the tubes.

Step 3: Colour Development Step: 500µlof substrate was added to tubes 1-100. The tubes were covered and were briefly vortex mixed. The tubes were then transferred to a 37^{o}C water bath and incubated for 30 minutes. After removing the tubes from the water bath, 1ml of EIA stop buffer was added to tubes 1-100 and briefly vortex mixed. The rack was then placed on a magnetic base for 10minutes.

The spectrophotometer was set at wave length of 450nm. It was zero by using the substrate blank solution (Tube 101). The OD at 450nm of solutions in tubes 1-100 were then measured determined.

The same procedures as enumerated above were employed for serum progesterone and estrogen estimation using individual kits. Concentrations were then read from standard curve.

3.53 Statistical analysis:

Mean values and standard error of mean (Mean ± SEM) were calculated. The test of significance between two groups was by Student's T test (Snedecor and Cochran, 1980) and for more than two groups by the analysis of variance (ANOVA) with Duncan's multiple range test (Duncan, 1975)

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical analysis on *Ricinus communis* oil

As shown in Table 4.1 physicochemical analysis of *Ricinus communis* oil gave an acid value of 0.154mgKOH/g, saponification value of 139.7, percentage freefatty acid value of 0.077, density of 0.95gm/ml at 25^0C and a pale yellow viscous liquid.

Table 4.1: The table below shows chemical and physical properties of *Ricinus communis* oil extracted from the seed

ACID VALUE	0.154
SAPONIFICATION VALUE	139.7
FREE FATTY ACID VALUE	0.077 mgKOH/g
DENSITY	0.95g/ml AT 20^0C
SOLUBILITY	IN WATER(<1MG/ML AT 20^0C;IN METHANOL(MISCIBLE)
COLOUR	PALE YELLOW VISCOUS LIQUID

4.2 Gas chromatography and molecular spectroscopy on *Ricinus communis* oil

Figure 4.1 showed the results of the gas chromatography and molecular spectroscopy (GCMS) of fixed oil isolated from the *Ricinus communis* seed showing the relative abundance of the chemical constituents with retention time. Overall, four major constituents were identified in the fixed oil; 5.90% of 9, 12 octadecadienic acid at retention time of 15.369s, 12.99% of 9,17 octadecadienal at retention time of 15.666s, 46.68% of 9 octa 12 hydroxydecanoic acid at retention time of 16.626s and finally 34.41% of ricinoliec acid at 17.049s retention time (Figures 4.2 and 4.3). Individual constituents of the oil were identified on the basis of their retention indices determined with a reference to a homologous series of n-alkanes and by comparison of their mass spectral fragmentation patterns. Fig4.4 showed the fragmentation pattern and structure of ricinoliec acid respectively.

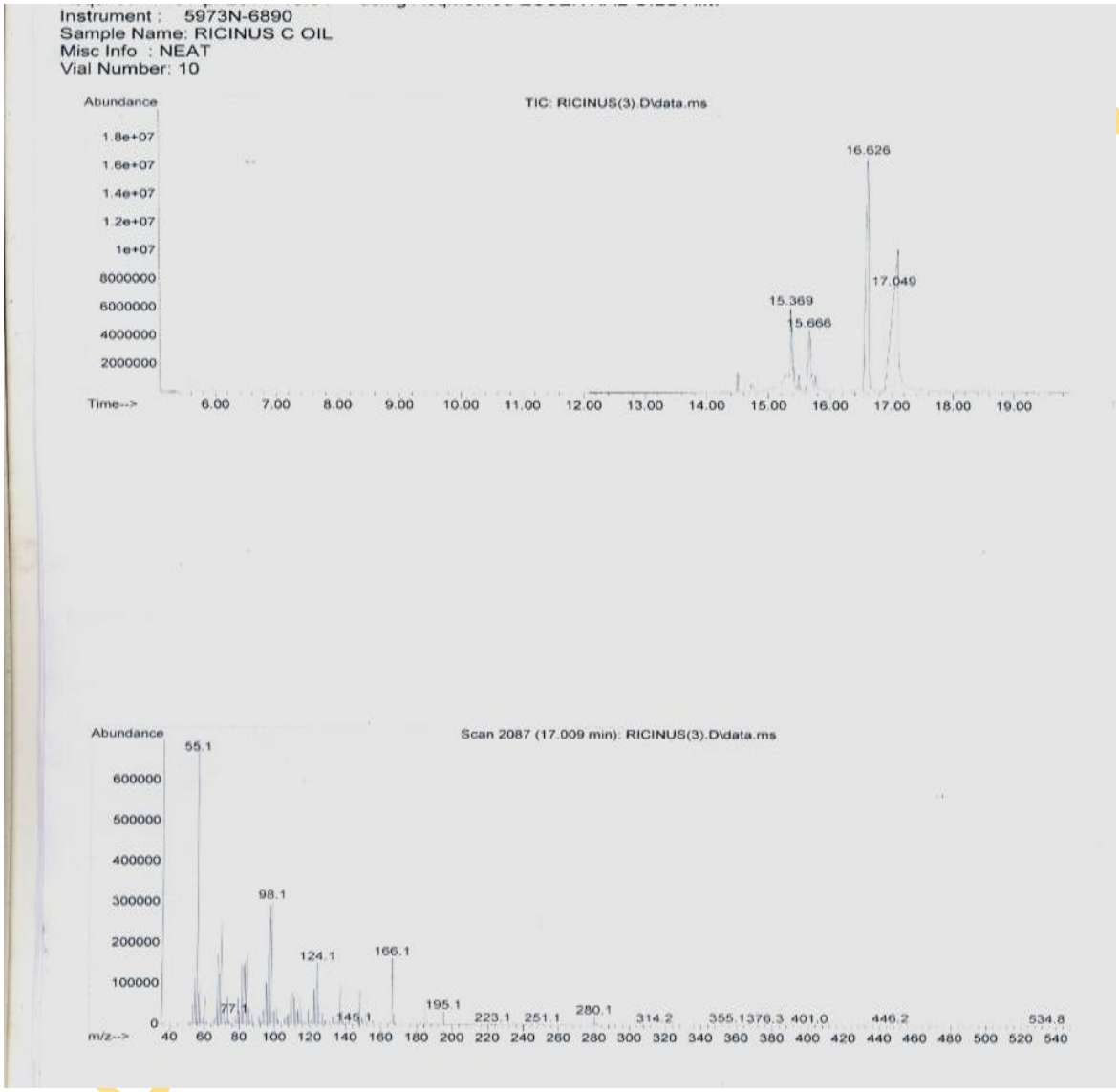
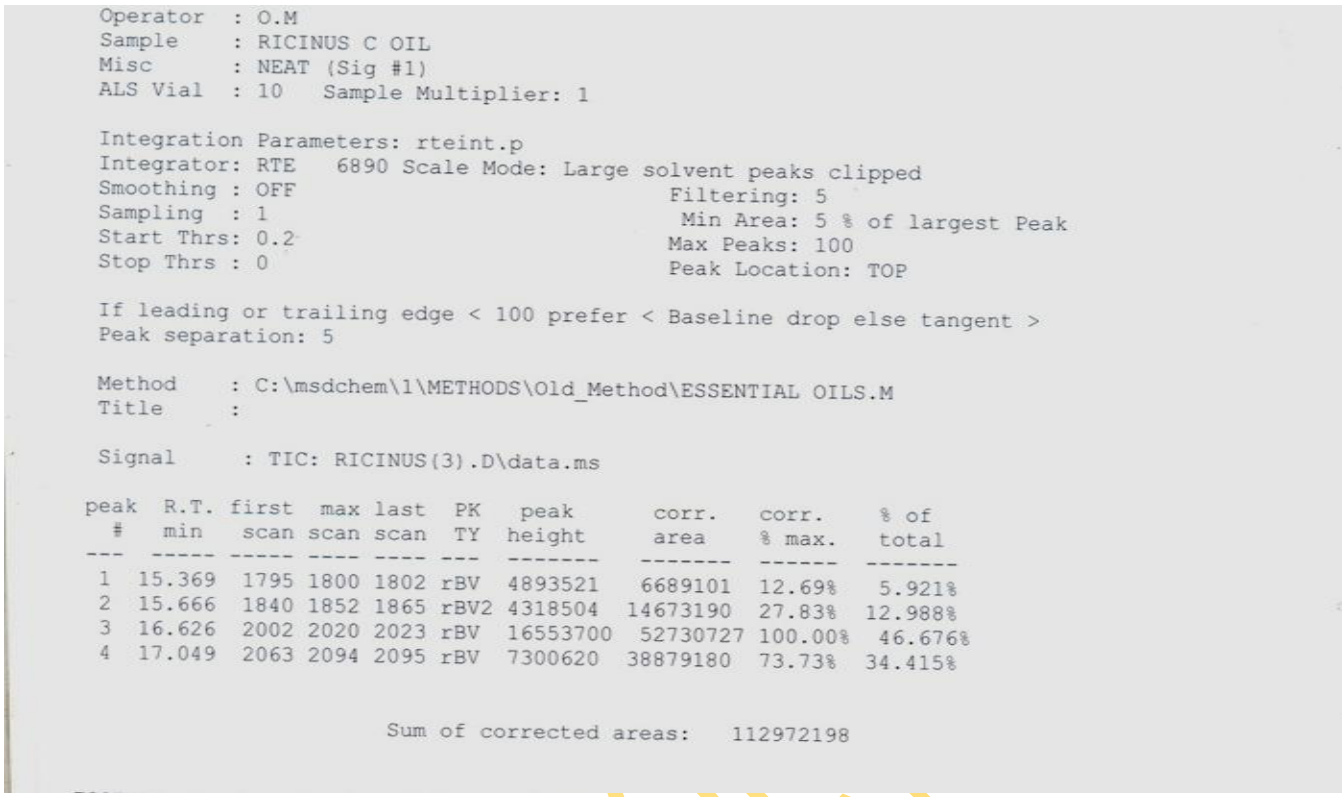
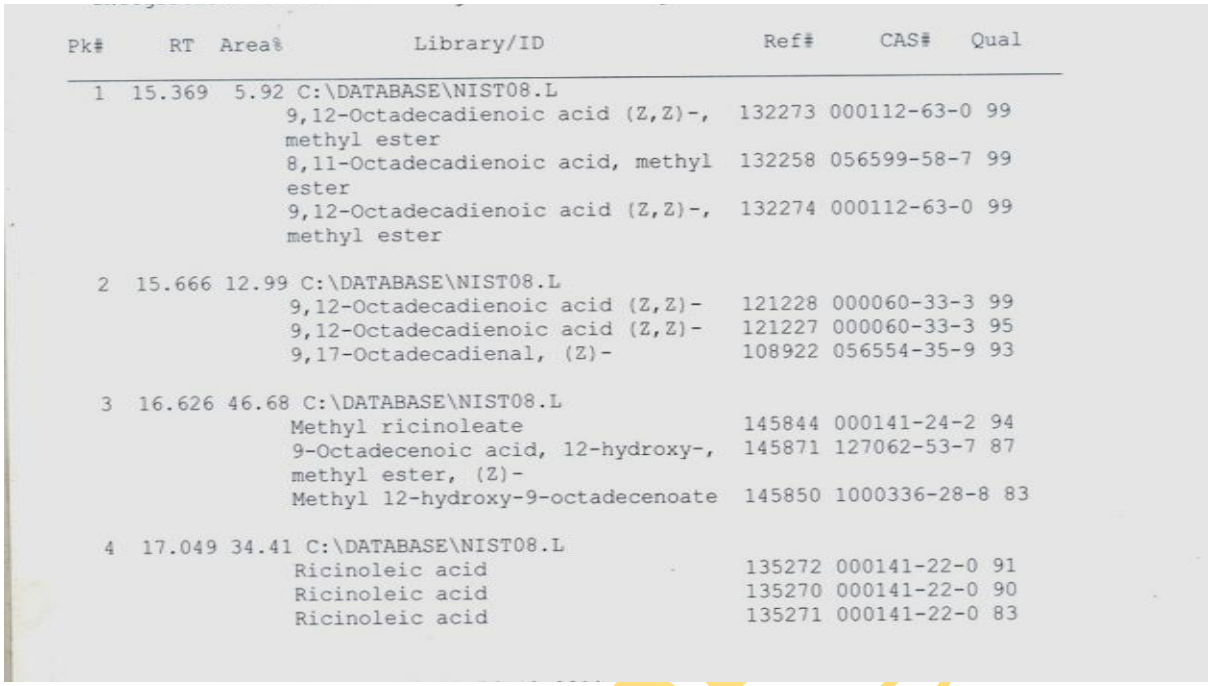


Figure 4.1: Gas chromatography and molecular spectroscopy on ***Ricinus communis*** **oil.**



```
Operator  : O.M
Sample    : RICINUS C OIL
Misc      : NEAT (Sig #1)
ALS Vial  : 10   Sample Multiplier: 1

Integration Parameters: rteint.p
Integrator: RTE   6890 Scale Mode: Large solvent peaks clipped
Smoothing : OFF                          Filtering: 5
Sampling  : 1                             Min Area: 5 % of largest Peak
Start Thrs: 0.2                          Max Peaks: 100
Stop Thrs : 0                            Peak Location: TOP

If leading or trailing edge < 100 prefer < Baseline drop else tangent >
Peak separation: 5

Method    : C:\msdchem\1\METHODS\Old_Method\ESSENTIAL OILS.M
Title     :

Signal     : TIC: RICINUS(3).D\data.ms

peak  R.T. first  max last  PK   peak      corr.    corr.    % of
  #    min  scan scan scan  TY  height     area     % max.   total
---  ----- ----- ---- ---- ---  -------   -------  ------  -------
 1  15.369  1795 1800 1802 rBV  4893521   6689101  12.69%   5.921%
 2  15.666  1840 1852 1865 rBV2 4318504  14673190  27.83%  12.988%
 3  16.626  2002 2020 2023 rBV  16553700  52730727 100.00%  46.676%
 4  17.049  2063 2094 2095 rBV  7300620  38879180  73.73%  34.415%


                    Sum of corrected areas:   112972198
```

Figure 4.2: Retention time and percentage of total constituents of *Ricinus communis* oil



Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	15.369	5.92	C:\DATABASE\NIST08.L			
			9,12-Octadecadienoic acid (Z,Z)-, methyl ester	132273	000112-63-0	99
			8,11-Octadecadienoic acid, methyl ester	132258	056599-58-7	99
			9,12-Octadecadienoic acid (Z,Z)-, methyl ester	132274	000112-63-0	99
2	15.666	12.99	C:\DATABASE\NIST08.L			
			9,12-Octadecadienoic acid (Z,Z)-	121228	000060-33-3	99
			9,12-Octadecadienoic acid (Z,Z)-	121227	000060-33-3	95
			9,17-Octadecadienal, (Z)-	108922	056554-35-9	93
3	16.626	46.68	C:\DATABASE\NIST08.L			
			Methyl ricinoleate	145844	000141-24-2	94
			9-Octadecenoic acid, 12-hydroxy-, methyl ester, (Z)-	145871	127062-53-7	87
			Methyl 12-hydroxy-9-octadecenoate	145850	1000336-28-8	83
4	17.049	34.41	C:\DATABASE\NIST08.L			
			Ricinoleic acid	135272	000141-22-0	91
			Ricinoleic acid	135270	000141-22-0	90
			Ricinoleic acid	135271	000141-22-0	83

Figure 4.3: Nomenclature of individual constituents for *Ricinus communis* oil

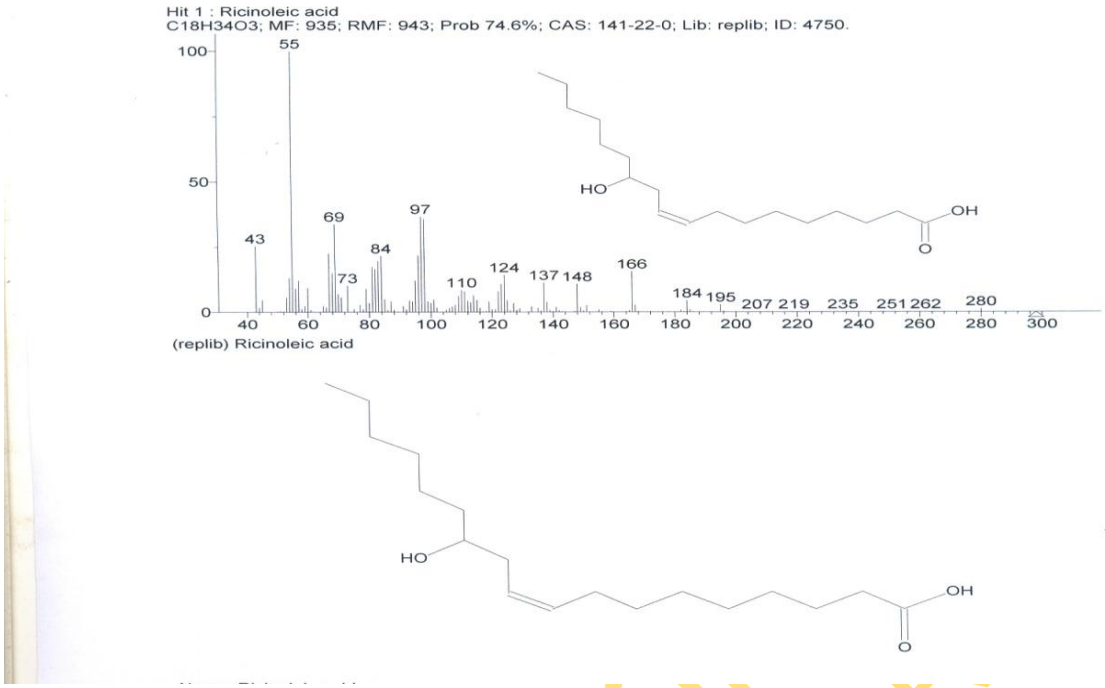


Figure 4.4: Fragmentation pattern and structure of ricinoliec acid

4.3 Acute oral toxicity study:

After more than 14days observatory period, there were no deaths or visible physical damage. Animals were without any visible and identifiable side effects or mortality even at 5000mg/kg bwt. Female rats were used though the experiment was also repeated in male rats. The result obtained for both sexes was the same. Studies have identified the fact that female rats are more susceptible to effects of chemicals than male rats (OECD, 425) and this study has bias to female because pregnant female rats were dosed (Table 4.2).

Table 4.2: **The table below shows the acute oral toxicity effect of administering *Ricinus communis* oil at 2000mg/kg body weight and 5000mg/kg body weight**

NO OF ANIMAL(FEMALE)	SURVIVAL	DEATH
1ST	100%	0%
2ND	100%	0%
3RD	100%	0%
4TH	100%	0%
5TH	100%	0%

4.4 Effect of maternal exposure to *Ricinus communis* oil on gestational weight in pregnant rats:

There were no significant differences in the mean weights of pregnant rats (Table 4.3) treated with *Ricinus communis* oil at different gestation period when compared with control for the three weeks gestation period.

Table 4.3: Body weight in treated pregnant rats dosed with *Ricinus communis* oil at different gestation periods

Groups(By gestation days) N= 5	Body weight(g)	Body weight(g)	Body weight(g)
	1st Week	2nd Week	3rd Week
CONTROL	204±10.30	219±14.18	232±13.93
GD 1-7	202±11.14	227±12.61	250±12.65
GD 7-14	200±7.07	229±13.64	240±20.98
GD 14-21	202±11.14	223±10.44	238±16.25
GD 1-21	202±13.93	213±12.41	230±14.14

4.5 Effects of maternal exposure to *Ricinus communis* oil on heamatological indices

As shown in Table 4.4, pregnant rats treated at gestation days 1-7 showed a reduced packed cell volume when compared with control, while pregnant rats treated on gestation days 7-14, 14-21 and 1-21 showed an increase in packed cell volume. The increase was however not significantly different from the control.

Table 4.4: Effects of *Ricinus communis* oil (950mg/kg B.W.) administered to pregnant female rats at different gestation period on packed cell volume, heamoglobin concentration and red blood cell count

Groups(By gestation days) **N=5**	**PCV (%)**	**HB(g/dl)**	**RBC(x10$^{12/}$/L)**
CONTROL	37.0±1.8	12.0± 0.7	5.98± 0.3
GD 1-7	35.4± 4.3	11.3± 0.7	5.77± 0.7
GD 7-14	38.6± 2.2	12.5± 0.8	6.36± 0.4
GD 14-21	39.4± 3.4	12.6± 1.3	6.54± 0.6
GD 1-21	39.0±0.9	12.3± 0.3	6.56±0.2

4.6 Effects of maternal exposure to *Ricinus communis* oil on leucocytes cells

There was an increase in the monocytes level as compared to control in pregnant rats treated with *Ricinus communis* oil at gestation days 1-7,7-14 and 1-21 (Table 4.5),while there was a decrease in the monocytes level in pregnant rats treated with *Ricinus communis* oil between gestation days 14-21(Table 4.5). Eosinophil levels in treated pregnant rats at gestation days 1-7, 7-14, 14-21 and 1-21 showed a decrease (Table 4.5). However, all changes were not statistically different from control. There were no significant differences from control, in the white blood cell count, lymphocyte count and neutrophil count in pregnant rats treated with *Ricinus communis* oil at different gestation periods (Table 4.5).

Table 4.5: Effects of *Ricinus communis* oil (950mg/kg B.W.) administered to pregnant female rats at different gestation period on leucocyte values

PARAMETERS	CONTROL (N=5)	GD 1-7 (N=5)	GD 7-14 (N=5)	GD 14-21 (N=5)	GD 1-21 (N=5)
TOTAL WBC($x10^9$/L)	8.2±4.8	7.7±9.9	8.8±3.5	8.6±15.9	8.1±15.8
LYMPHOCYTE ($x10^9$/L)	5.4±6.6	5.7±4.2	5.4±6.2	6.4±2.5	4.9±5.6
NEUTROPHIL ($x10^9$/L)	2.6±6.9	1.8±3.9	3.3±6.4	2.1±3.0	3.0±5.6
EOSINOPHIL ($x10^9$/L)	0.1±0.2	0.0±0.2	0.0±0.4	0.0±0.2	0.1±0.4
MONOCYTE ($x10^9$/L)	0.1±0.7	0.2±0.7	0.2±0.8	0.1±0.5	0.2±0.4

4.7 Effects of maternal exposure to *Ricinus communis* oil on serum alanine aminotransferase

There was a significant decrease ($p<0.05$) when compared to the control in serum level of alanine aminotransferase in pregnant rats treated with *Ricinus communis* oil between gestation days 7-14 and 14-21 (Fig4.5). Serum level of alanine aminotransferase increased in pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, and 1-21, the increase (Fig4.5) was however not statistically different when compared with the control.

4.8 Biochemical parameters in pregnant rats treated with *Ricinus communis* oil at different gestation period

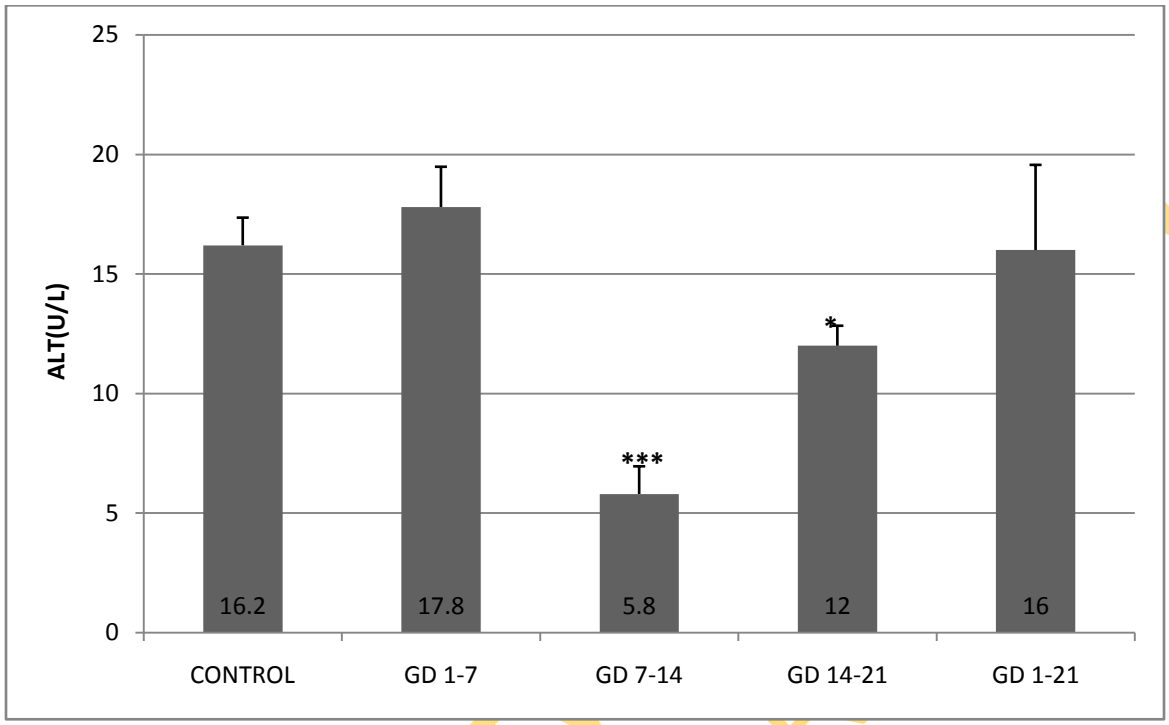


Figure 4.5: Serum alanine aminotransferase (ALT), level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05, ***=p< 0.001

4.9 Effects of maternal exposure to *Ricinus communis* oil on serum aspartate aminotransferase

There were statistically significant decreases from control (Fig.4.6) in the serum aspartate aminotransferase levels of pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, 7-14, and 1-21($p<0.01$). Pregnant rats treated with *Ricinus communis* oil between gestation days 14-21 also showed decrease in serum aspartate aminotransferase. The decrease was however not statistically different from the control.

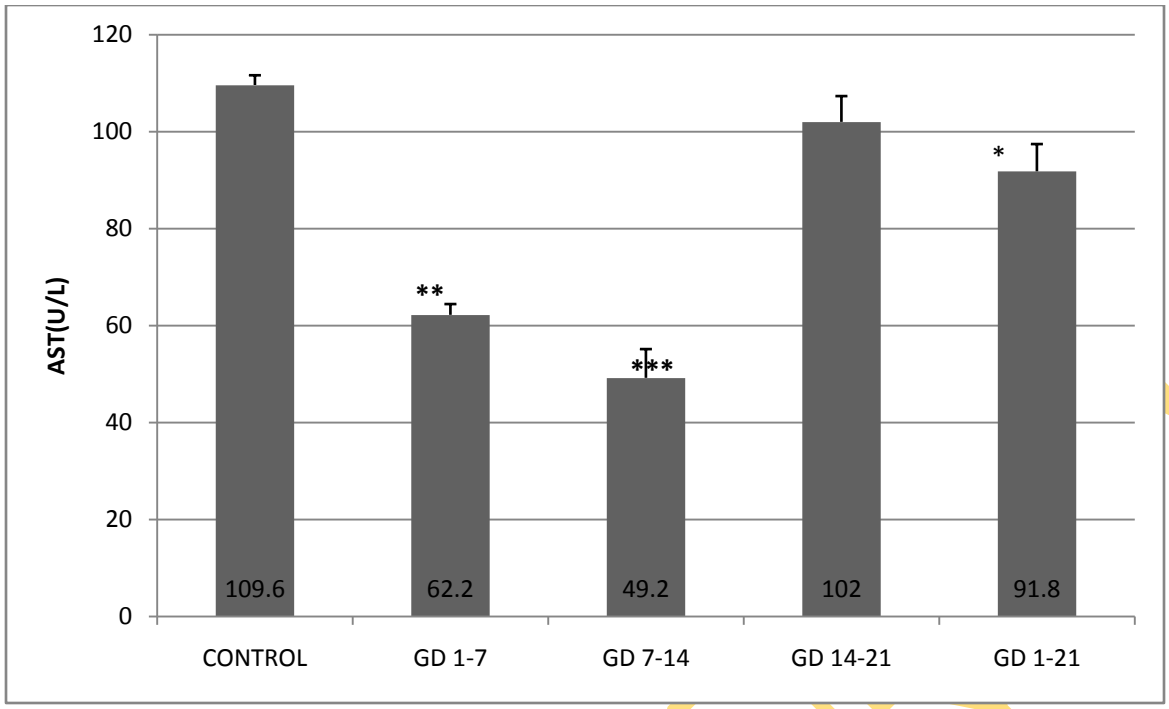


Figure 4.6: Serum aspartate aminotransferase (AST) level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p < 0.05, ** =p< 0.01, *** =p< 0.001

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4.10 Effects of maternal *Ricinus communis* oil exposure on serum cholesterol level

There were statistically significant increases (Fig.4.7) in the serum levels of total cholesterol in pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, 7-14, 14-21 and 1-21 ($p < 0.01$).

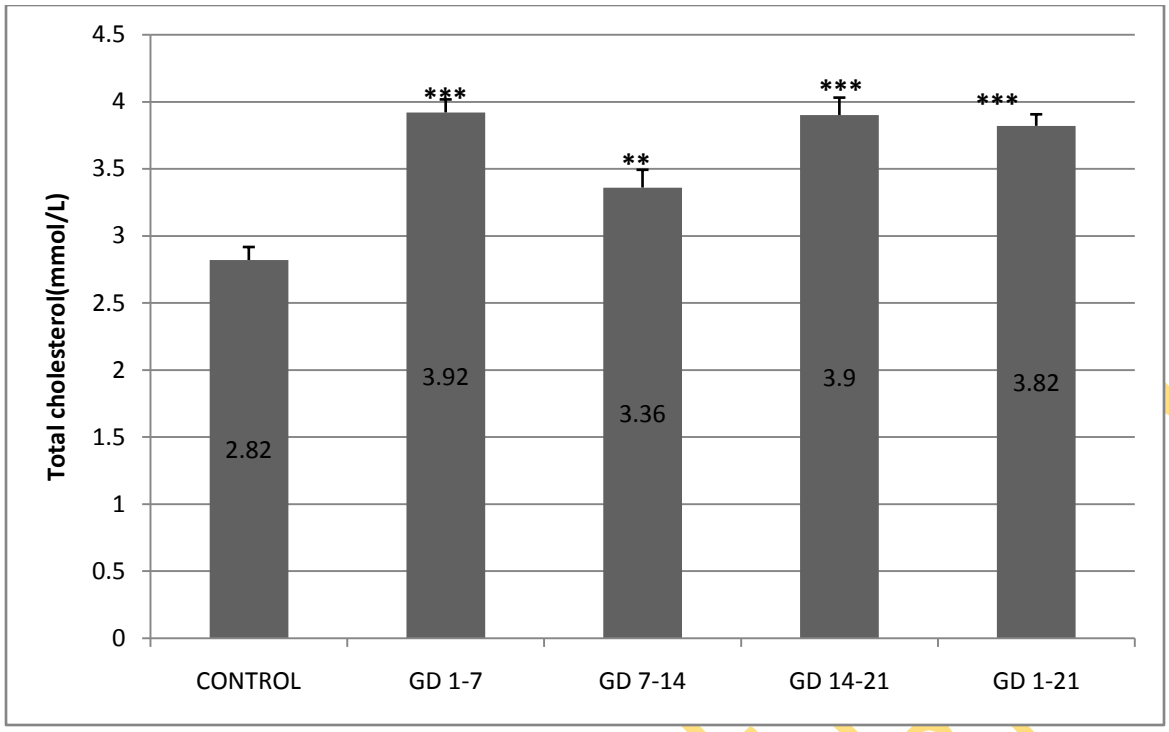

Figure 4.7: Serum total cholesterol level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: ** =p< 0.01, ***=p< 0.001

4.11 Effects of maternal *Ricinus communis* oil exposure on serum triglyceraldehyde content

As shown in Figure 4.8 there were statistically significant increases when compared with the control in the serum triglyceraldehyde content in pregnant rats treated with *Ricinus communis* oil at gestation days 1-7, 7-14, 14-21 and 1-21 ($p<0.01$).

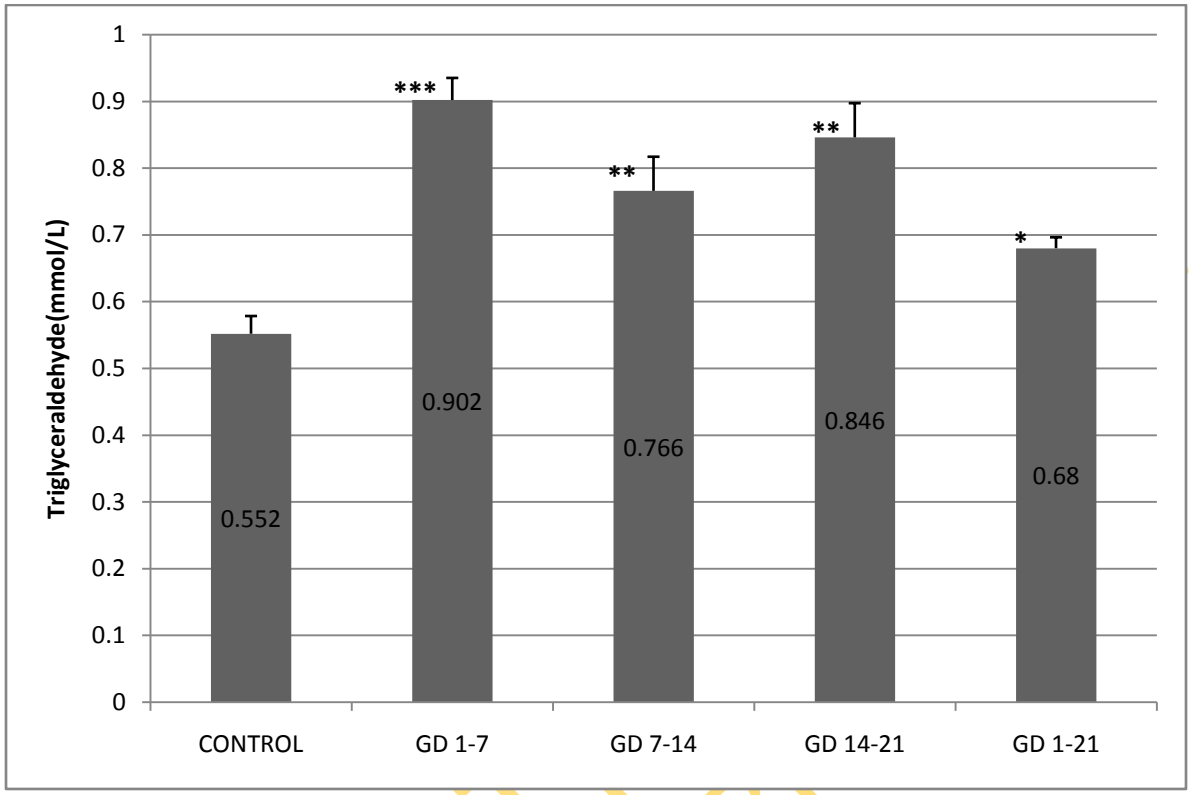

Figure 4.8: Serum triglyceraldehyde content in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05, * =p< 0.01, *** =p< 0.001

4.12 Effects of maternal exposure to *Ricinus communis* oil on serum high density lipoprotein level

There were also statistically significant increases when compared with the control (Fig.4.9) in the serum high density lipoprotein level in pregnant rats treated with *Ricinus communis* oil at gestation days 1-7, 7-14, 14-21 and 1-21 ($p<0.05$ and 0.01).

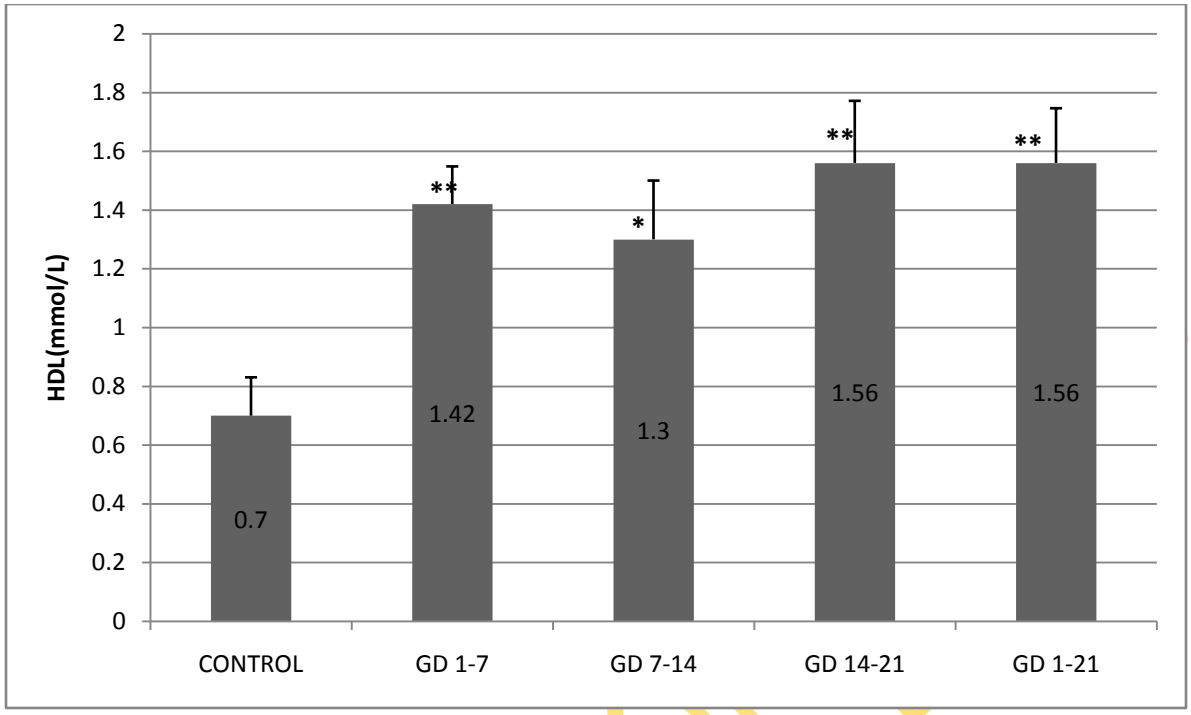

Figure 4.9: Serum high density lipoprotein (HDL) level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05, **=p< 0.01

4.13 Effects of maternal exposure to *Ricinus communis* oil on serum low density lipoprotein

There was a statistically significant decrease when compared with the control (Fig.4.10) in serum low density lipoprotein levels in pregnant rats treated with *Ricinus communis* oil between gestation days 7-14 ($p<0.05$). However, increases in low density lipoprotein levels were not statistically significant for pregnant rats treated between gestation days 1-7, 14-21 and 1-21 (Fig 4.10).

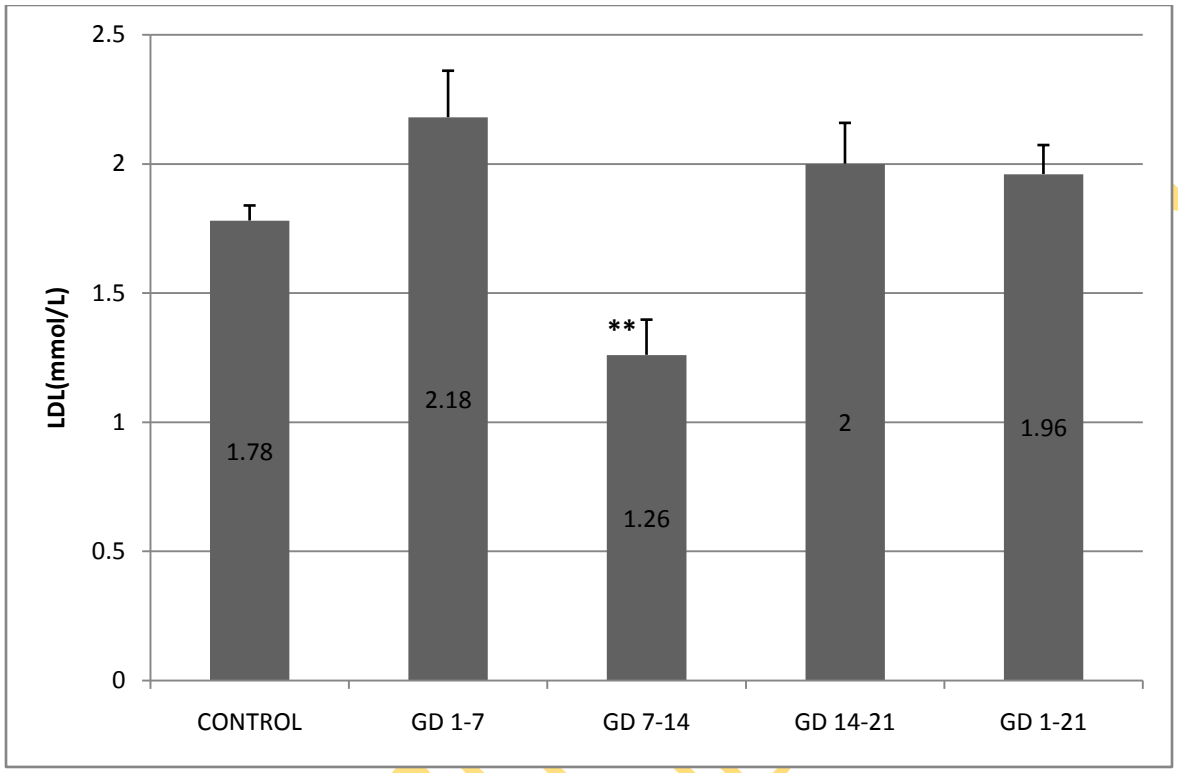

Figure 4.10: Serum low density lipoprotein (LDL) level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: ** =p< 0.01

4.14 Effects of maternal exposure to *Ricinus communis* oil on serum progesterone level

There were significant decreases ($p<0.05$) in the progesterone levels in all *Ricinus communis* oil treated groups (Fig.4.11).

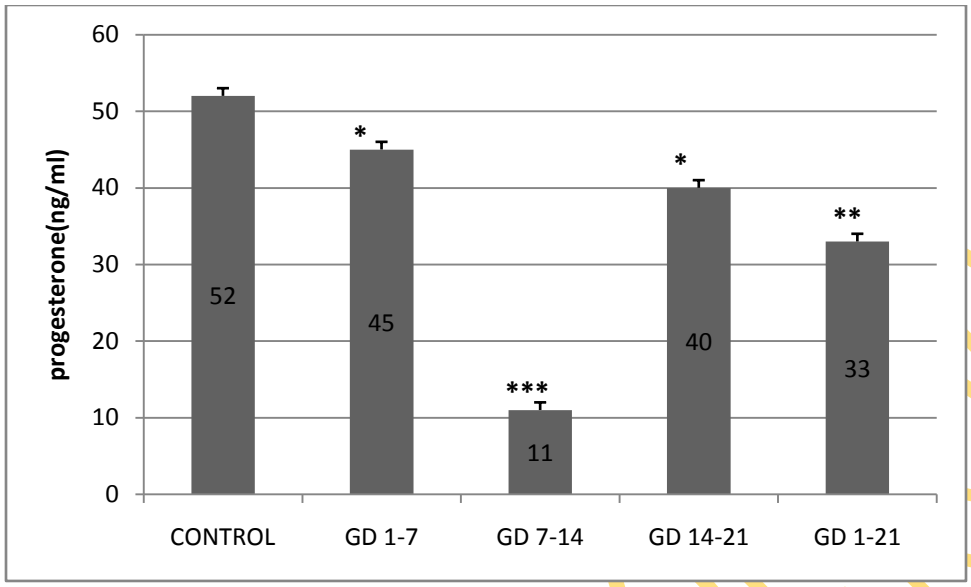

Figure 4.11: Serum progesterone level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05, ** =p< 0.01, *** =p< 0.001

4.15 Effects of maternal exposure to *Ricinus communis* oil on serum estrogen level

Serum estrogen levels significantly ($p<0.05$) reduced in all *Ricinus communis* oil treated rats when compared with the control (Fig. 4.12).

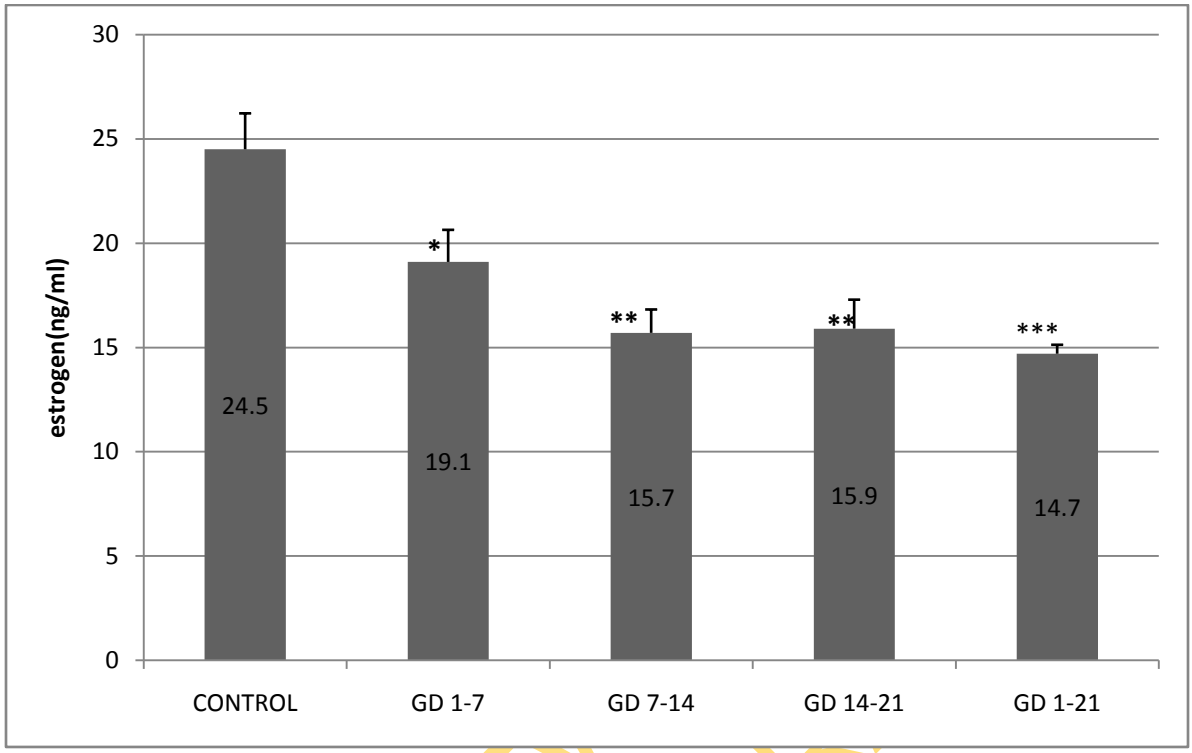

Figure 4.12: Serum estrogen level in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05, ** =p< 0.01, *** =p< 0.001

4.16 Effects of maternal exposure to *Ricinus communis* oil on mean organ weights of treated pregnant rats

As shown in Table 4.6, there were no significant changes in the mean organ weight between treated pregnant rats and untreated pregnant control.

Table 4.6: Organ weight in pregnant rats treated with *Ricinus communis* oil (950mg/kg BW) at different gestation periods (GD= gestation days)

ORGANS(g)	CONTROL (N=5)	GD 1-7 (N=5)	GD 7-14 (N=5)	GD 14-21 (N=5)	GD 1-21 (N=5)
OVARY	0.03±0.00	0.02±0.07	0.03±0.00	0.03±0.01	0.06±0.01
UTERUS	0.09±0.02	0.02±0.07	0.14±0.02	0.07±0.02	0.13±0.03
KIDNEY	0.28±0.03	0.26±0.01	0.30±0.01	0.30±0.07	0.22±0.01
LIVER	3.80±0.60	3.10±0.10	3.30±0.20	3.60±0.40	3.00±0.20

4.17 Effects of maternal exposure to *Ricinus communis* oil on histology of the ovary and uterus of treated rats

There were no lesions in the ovary of female rats from control and *Ricinus communis* oil treated groups (plates 4.1, 4.2, 4.3, 4.4 and 4.5). Uterus of female rats from gestation days 7-14 showed ballooning of the uterine epithelia cells (plate 4.8) and implantation sites with resorptions for female rats from GD 1-7 (plate 4.7) and 14-21, (plate 4.9).

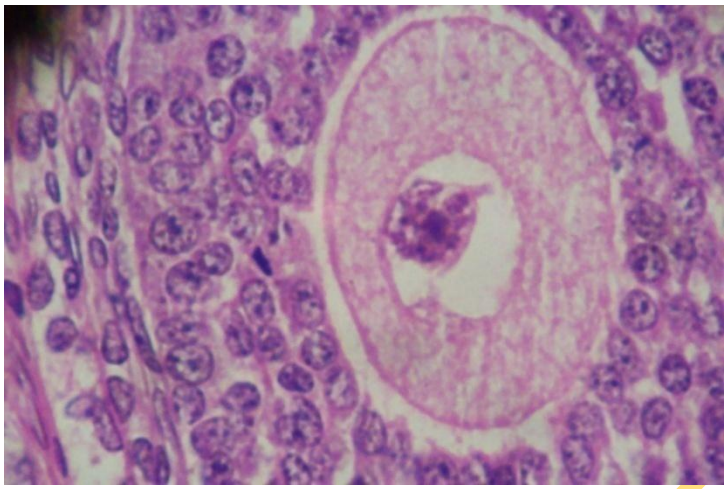

Plate 4.1: Photomicrograph of the ovary from control pregnant rats, no visible lesion seen (X400)

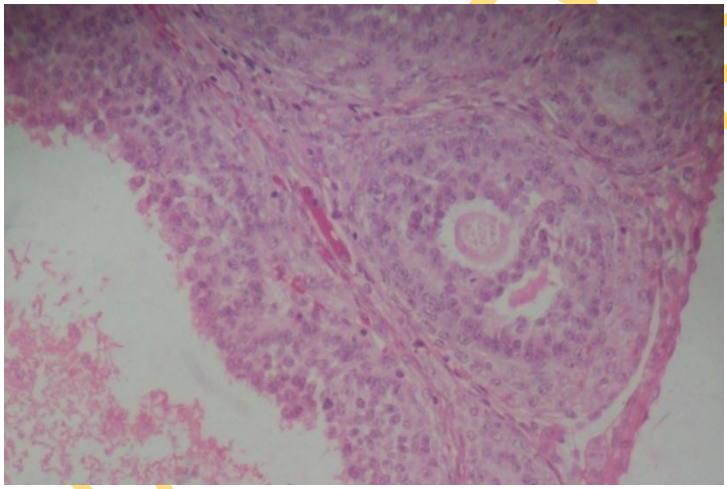

Plate 4.2: Photomicrograph of the ovary from pregnant rats exposed between gestation days (GD) 1-7. No visible lesion seen. (X400)

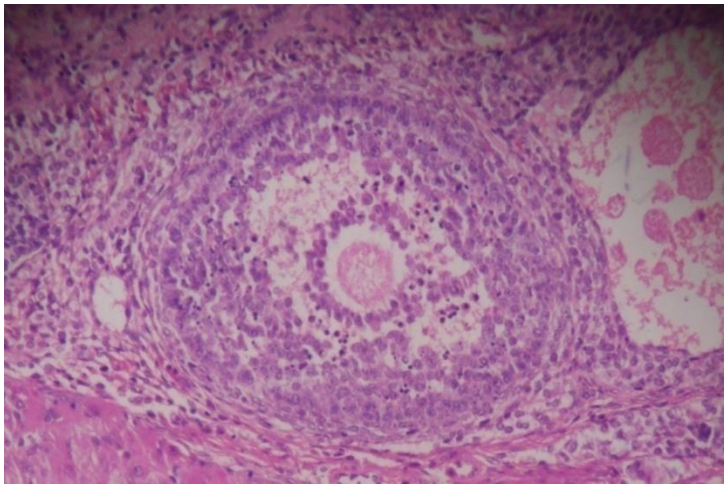

Plate 4.3: Photomicrograph of the ovary from pregnant rats exposed between gestation days (GD) 7-14. No visible lesion seen (X400)

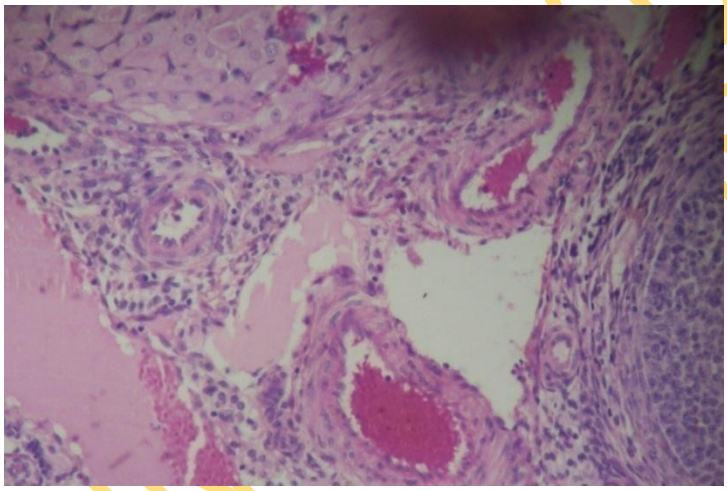

Plate 4.4: Photomicrograph of the ovary from pregnant rats exposed between gestation days (GD) 14-21. No lesions seen (X400)

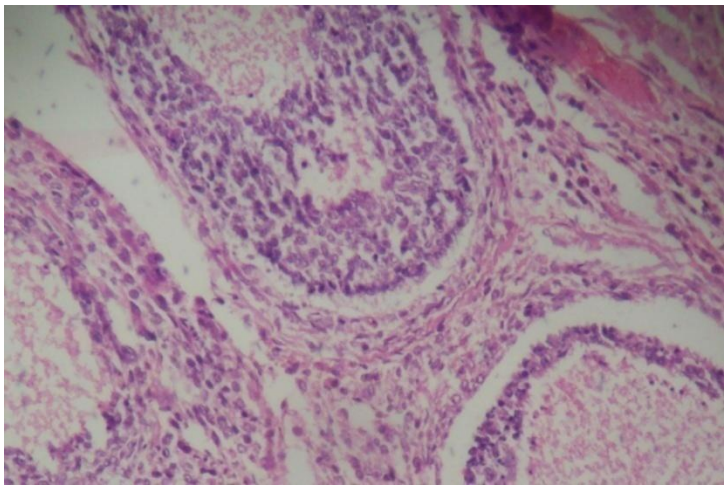

Plate 4.5: Photomicrograph of the ovary from pregnant rats exposed between gestation days (GD) 1-21. No visible lesion seen (X400)

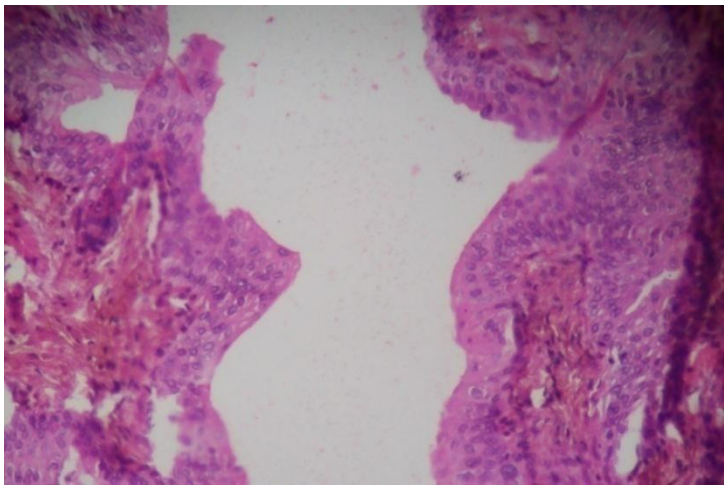

Plate 4.6: Photomicrograph of the uterus from control pregnant rat (X400), no visible lesion seen.

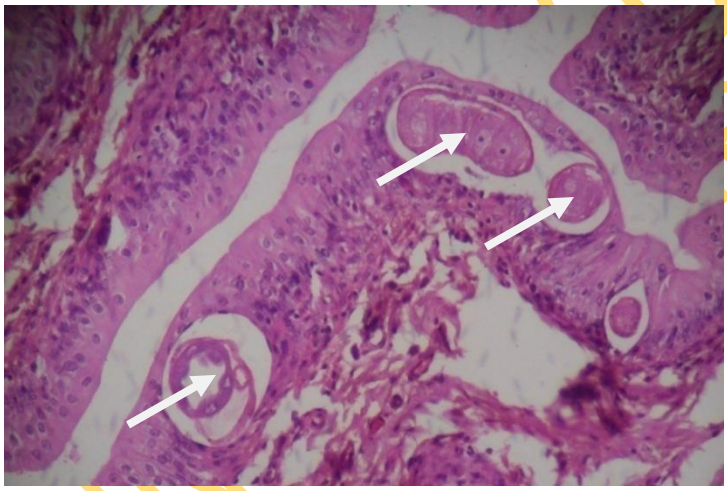

Plate 4.7: Photomicrograph of the uterus from pregnant rats exposed between gestation days (GD) 1-7. Implantation sites present but no lesions seen (X400)

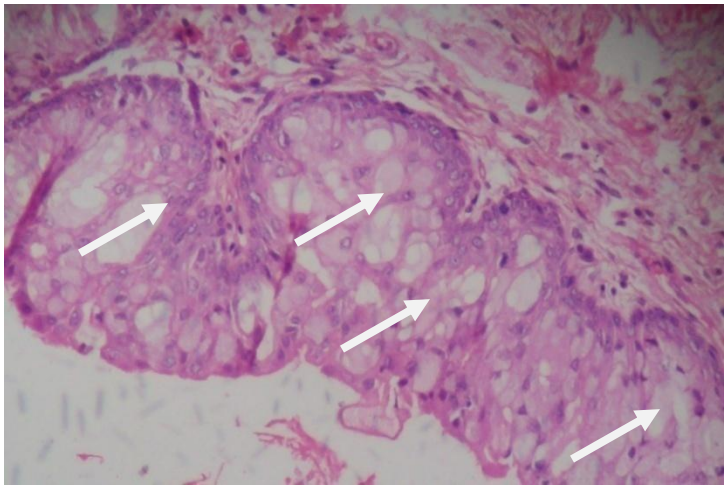

Plate 4.8: Photomicrograph of the uterus from pregnant rats exposed between gestation days (GD) 7-14. There is ballooning of some of the epithelial cells. (x400)

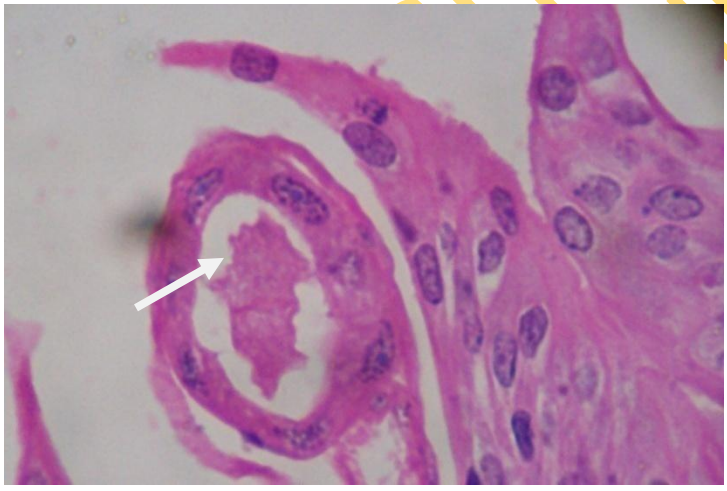

Plate 4.9: Photomicrograph of the uterus from pregnant rats exposed between gestation days (GD) 14-21. No lesions seen, fewer implantation sites with foci (X400)

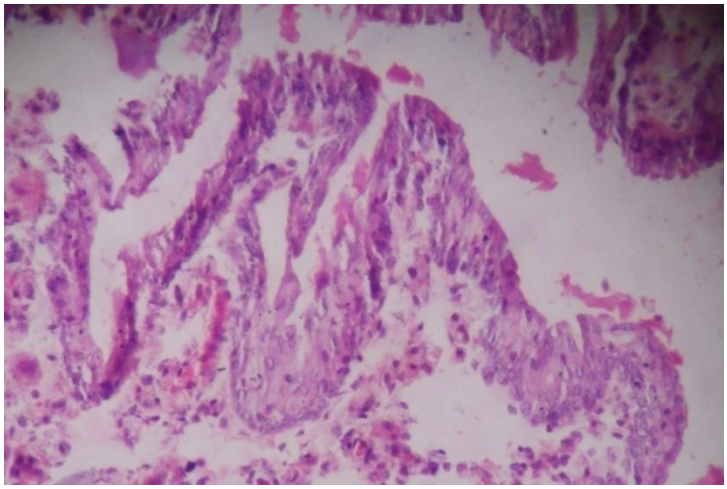

Plate 4.10: Photomicrograph of the uterus from pregnant rats exposed between gestation days (GD) 1-21. No visible lesion seen (X400)

4.18 Effects of maternal exposure to *Ricinus communis* oil at different gestation periods on litter outcome

There were no significant decreases in the number of average litter when compared to the control in pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, 7-14, and 1-21, (Fig.4.13). Litters from gestation days 14-21 however showed significant decrease when compared with the control, ($p<0.05$), (Fig.4.13).

4.19 Average number of F_1 pups at birth after maternal exposure to R*icinus communis* oil at different gestation period

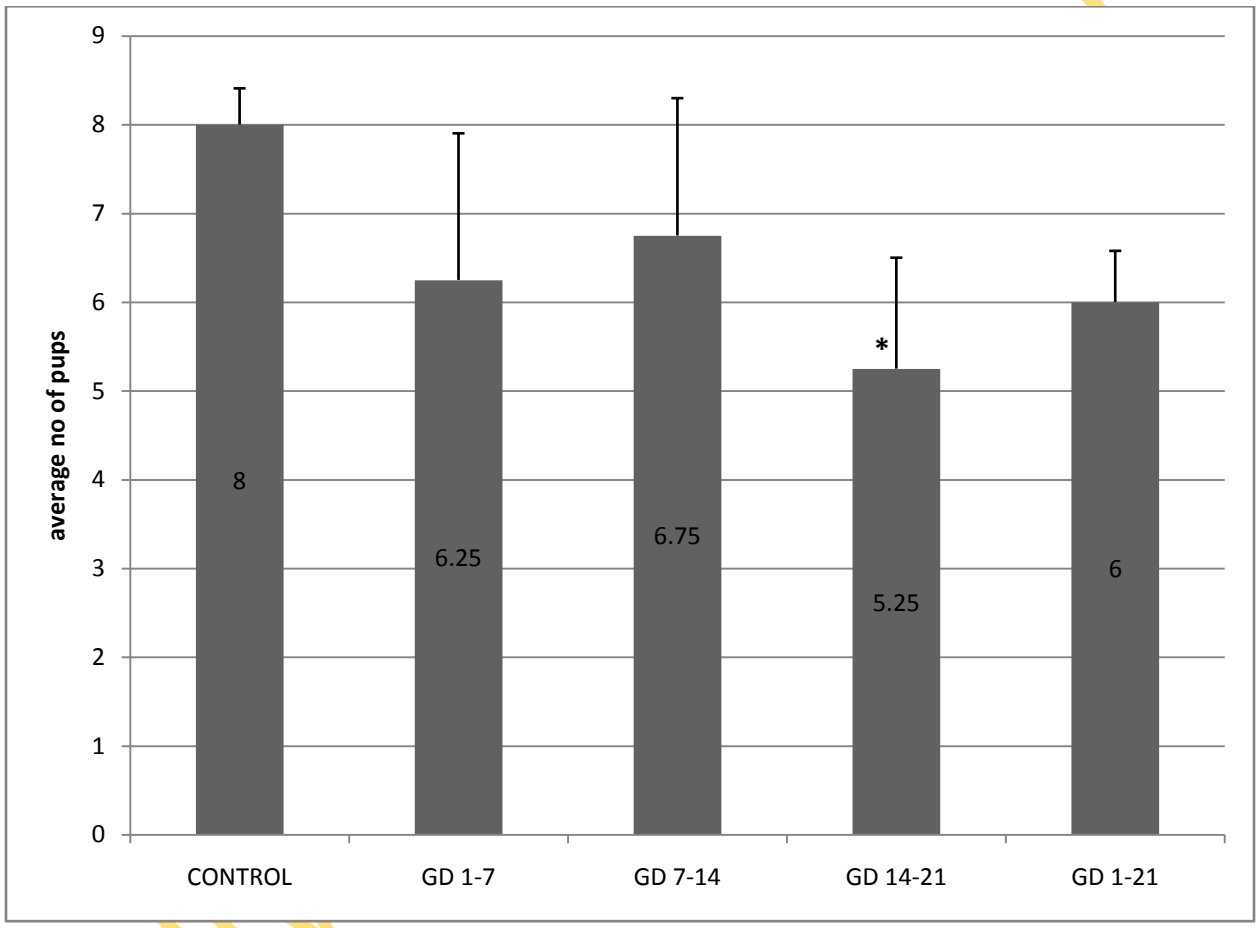

Figure 4.13: Average litter size per group in pregnant rats treated with *Ricinus communis* oil at different gestation periods

KEY: * = p< 0.05.

4.20 Effects of maternal exposure to *Ricinus communis* oil on morphometric data of F_1 female pups

As shown in Table 4.7, F_1 female pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7 showed significant decrease in weight when compared with the control ($p<0.05$). F_1 female pups from treated pregnant rats between gestation days 7-14, 14-21 and 1-21 did not show any significant difference in weigh (Table. 4.7).

There were no significant changes in the head diameter when compared with the control of F_1 female pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, 7-14, and 14-21, however, female pups from gestation days 1-21 showed significant when compared with the control (Table 4.7).

There were no significant changes (Table 4.6) in abdominal diameter of F_1 female pups from pregnant rats treated with *Ricinus communis* oil at gestation days 1-7, 7-14, 14-21, and 1-21. Similarly there were no significant changes in body length when compared with control in F_1 Female pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7, 7-14, and 14-21, (Table 4.7). Female pups from gestation days 1-21 treated rats showed significant increase in body length when compared with the control ($p<0.05$).

Table 4.7: Morphometric data in F_1 female pups at birth (PND 1) from pregnant rats treated with *Ricinus communis* oil at different gestation periods

GROUPS BY GESTATION DAY(N=7)	WEIGHT(g)	HEAD DIAMETER(cm)	ABDOMINAL DIAMETER(cm)	BODY LENGHT(cm)
CONTROL	5.74±0.28	1.15±0.02	1.39±0.04	4.59±0.11
GD 1-7	5.00±0.09*	1.14±0.03	1.32±0.04	4.41±0.03
GD 7-14	5.35±0.12	1.12±0.01	1.44±0.03	4.62±0.09
GD 14-21	5.92±0.20	1.16±0.01	1.47±0.05	4.67±0.06
GD 1-21	5.89±0.11	1.24±0.03*	1.27±0.04	4.95±0.09*

KEY: * = p< 0.05

4.21 Effects of maternal exposure to *Ricinus communis* oil on morphometric data in F_1 male pups

There was a significant decrease (Table 4.8) in weight of F_1 male pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7 and 7-14 ($p<0.05$). However, the decreases observed in weight of F1 male pups from gestation days 7-14, 14-21 and 1-21 were not statistically significant.

Head diameter in F_1 male pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7 and 7-14 showed significant decrease ($p<0.05$). There were however no significant decreases in head diameter in F_1 male pups from gestation days 14-21 and 1-21 (Table 4.8).

There was a significant decrease in abdominal diameter and body lenght in F_1 male pups from pregnant rats treated with *Ricinus communis* oil between gestation days 1-7 ($p<0.01$). There were however no significant decrease in abdominal diameter of male pups from rats treated at gestation days 7-14, 14-21 and 1-21 (Table 4.8).

Table 4.8: Morphometric data in F_1 male pups at birth (PND 1) from pregnant rats treated with *Ricinus communis* oil at different gestation periods.

GROUP BY GESTATION PERIOD(MALE) N=7	WEIGHT**(g)**	HEAD DIAMETER**(cm)**	ABDOMINAL DIAMETER**(cm)**	BODY LENGHT**(cm)**
CONTROL	6.49±0.45	1.24±0.04	1.52±0.04	5.05±0.18
GD 1-7	5.28±0.17**	1.15±0.02*	1.36±0.02**	4.53±0.08**
GD 7-14	5.64±0.17*	1.13±0.01**	1.43±0.04	4.67±0.12
GD 14-21	5.70±0.44	1.16±0.03	1.47±0.04	4.79±0.18
GD 1-21	6.28±0.17	1.19±0.01	1.46±0.02	4.84±0.04

KEY: * = p< 0.05, ** =p< 0.01

4.22 Effects of maternal exposure to *Ricinus communis* oil on anogenital distance and index in F_1 female pups

As shown in Table 4.9, there was statistically significant decrease of the anogenital distance in F_1 female pups from gestation days 7-14 treated rats ($p<0.05$). Decrease in the anogenital distance of female pups from gestation days 1-7, 14-21 and 1-21 were however not statistically significant. Anogenital index was significantly reduced in female pups from gestation days 14-21 treated rats ($p<0.05$).

Table 4.9: Anogenital distance at birth in F_1 female pups (PND 1) from pregnant rats treated with *Ricinus communis* oil at different gestation periods.

GROUP BY GESTATION DAY (N=7)	ANOGENITAL DISTANCE(cm)	ANOGENITAL DISTANCE INDEX(cm/gm^3)
CONTROL	0.22±0.01	0.12±0.01
GD 1-7	0.19±0.01	0.11±0.01
GD 7-14	0.18±0.02*	0.10±0.01
GD 14-21	0.19±0.02	0.11±0.01*
GD 1-21	0.24±0.01	0.13±0.01

KEY: * = p< 0.05

4.23 Effects of maternal exposure to *Ricinus communis* oil on anogenital distance and index in F_1 male pups

As shown in Table 4.10 there were significant decreases ($p<0.05$) in both the anogenital distance and anogenital indexes in all F_1 male pups from pregnant rats treated with *Ricinus communis* oil on gestation days 1-7, 7-14, 14-21 and 1-21.

Table 4.10: Anogenital distance at birth in F_1 male pups (PND 1) from pregnant rats treated with *Ricinus communis* oil at different gestation periods

GROUP BY GESTATION DAY ((N=8))	ANOGENITAL DISTANCE(cm)	ANOGENITAL DISTANCE INDEX(cm/gm^3)
CONTROL	0.46±0.01	0.25±0.01
GD 1-7	0.37±0.01**	0.21±0.01*
GD 7-14	0.39±0.02**	0.22±0.01*
GD 14-21	0.35±0.01***	0.20±0.01**
GD 1-21	0.44±0.39*	0.21±0.01*

KEY: * = p< 0.05, ** =p< 0.01, *** =p< 0.001

4.24 Effects of maternal exposure to *Ricinus communis* oil on weekly pup weight until weaning in F_1 female pups

Average weight significantly decreased ($p<0.05$) in female pups from pregnant rats treated with *Ricinus communis* oil at gestation days 1-7. Female pups from gestation days 14-21 treated rats showed significant increase when compared with the control in average weight at 1st and 3rd week, ($p<0.05$). Average weights at 1st and 3rd week were not significantly different from control in female pups from rats treated at gestation days 7-14 and 1-21 (Table 4.11).

Table 4.11: Weekly average body weight from birth until weaning at 3rd week (PND 21) in F_1 female pups from pregnant rats treated with *Ricinus communis* oil at different gestation periods.

GROUP BY GESTATION DAY (N=7)	1ST WEEK WEIGHT (g)	3RD WEEK WEIGHT(g)
CONTROL	11.20±0.32	25.90±0.89
GD 1-7	9.59±0.18***	20.60±0.54***
GD 7-14	12.61±0.57	25.38±0.99
GD 14-21	12.89±0.09**	30.78±0.28***
GD 1-21	12.02±0.35	26.21±0.29

KEY: ** =p< 0.01, *** =p< 0.001

4.25 Effects of maternal exposure to *Ricinus communis* oil on weekly pup weight until weaning in F_1 male pups

Table 4.12 showed that there was a significant decrease ($p<0.05$) in body weight at 1st and 3rd week in male pups from pregnant rats treated with *Ricinus communis* oil at gestation days 1-7. There was also a significant decrease ($p<0.05$) when compared with the control in body weight at 1st week but not at 3rd week in male pups from pregnant rats treated with *Ricinus communis* oil at gestation days 7-14. Body weights at 1st and 3rd week were not significantly different from control in male pups from gestation days 14-21 and 1-21.

Table 4.12: Weekly body weight from birth until weaning at 3rd week (PND 21) in F1 male pups from pregnant rats treated with *Ricinus communis* oil at different gestation periods

GROUP BY GESTATION DAY(N=7)	1ST WEEK WEIGHT (g)	3RD WEEK WEIGHT(g)
CONTROL	12.69±0.16	28.63±0.29
GD 1-7	9.82±0.24**	20.85±0.44**
GD 7-14	10.79±0.37**	28.33±1.52
GD 14-21	12.00±0.93	29.2±1.31
GD 1-21	11.71±0.31*	27.62±0.51

KEY: * = p< 0.05, ** =p< 0.01

4.26 Effects of maternal exposure to *Ricinus communis* oil on weight and puberty attainment in F_1 Female

There were no significant changes in the average weight on attainment of puberty (Table 4.13) in all female pups from rats treated with *Ricinus communis* oil at gestation days 1-7, 7-14, 14-21 and 1-21 ($p>0.05$). As shown in Table 4.13, there was significant decrease ($p<0.05$) in the average day it took to attain puberty in the female pups from rats treated at gestation days 7-14. Female pups from gestation days 1-7, 14-21 and 1-21 treated rats do not show significant difference in average day on which puberty was achieved.

Table 4.13: Weight and day on attainment of puberty in F_1 female pups from pregnant rats treated with *Ricinus communis* oil at different gestation periods.

GROUP BY GESTATION DAY (N=7)	WEIGHT(g)	PUBERTY ONSET (DAYS)
CONTROL	80.52±5.84	49.00±2.28
GD 1-7	79.31±3.77	49.25±1.94
GD 7-14	79.38±2.64	42.20±1.20*
GD 14-21	91.98±2.90	49.80±1.28
GD 1-21	89.00±2.72	52.00±0.55

KEY: * = p< 0.05

4.27 Effects of maternal exposure to *Ricinus communis* oil on weight and puberty attainment in F_1 male

There were decreases though not significantly different from the control in the average weight on attainment of puberty in F_1 male pups from rats treated with *Ricinus communis* oil at gestation days 7-14, 14-21, and 1-21 (Table 4.14). However, F_1 Male pups from rats treated at gestation days 1-7 showed significant decrease in average weight on attainment of puberty ($p<0.05$). There were decreases though not significant from control in the average day on attainment of puberty in F_1 males from rats in all treated groups (Table 4.14).

Table 4.14: Weight and day on attainment of puberty in F_1 male pups from pregnant rats treated with *Ricinus communis* oil at different gestation periods.

GROUP BY GESTATION DAY(N=5)	WEIGHT(g)	PUBERTYONSET(DAYS)
CONTROL	89.25±9.89	49.20±4.16
GD 1-7	65.49±4.03*	47.00±2.27
GD 7-14	75.05±5.40	47.40±3.43
GD 14-21	90.18±9.52	44.40±3.10
GD 1-21	96.42±8.22	48.20±2.94

KEY: * = p< 0.05

4.28 Body weight and reproductive parameters of adult F_1 male and female

4.29 Effects of maternal exposure to *Ricinus communis* oil on weight, anogenital distance and index in adult F_1 male at sacrifice.

As shown in Table 4.15, F_1 adult male rats from gestation days 1-7, and 7-14 decreased significantly ($p<0.05$) in weight at sacrifice on postnatal day 90. However decrease in weight of F_1 male rats from gestation days 14-21 and 1-21 were not statistically significant. Absolute anogenital distance decreased significantly ($p<0.01$) in all F_1 males from treated female. Measure of anogenital index was however only significantly reduced ($p<0.05$) in F_1 males from rats treated at gestation days 1-7. Reductions in anogenital indexes of F_1 males from gestation days 7-14, 14-21 and 1-21 were not significant.

Table 4.15: Weights and anogenital distance of F_1 males at sacrifice (PND 90)

GROUP BY GESTATION DAY(N= 8)	WEIGHT (g)	AGD (cm)	AGD INDEX(cm/gm^3)
CONTROL	234±5.10	3.77±0.04	0.61±0.01
GD 1-7	182±4.90***	3.21±0.11**	0.56±0.02*
GD 7-14	194±6.80**	3.50±0.15*	0.59±0.03
GD 14-21	223±1.90	3.64±0.03*	0.59±0.01
GD 1-21	224±8.70	3.48±0.11*	0.57±0.02

KEY: * = p< 0.05, **=p< 0.01, ***=p< 0.001

4.30 Effects of maternal exposure to *Ricinus communis* oil on absolute organs weight in adult F_1 male

As shown in Table 4.16 there was significant decrease ($p<0.05$) in the absolute weight of the testis in F_1 male rats from rats treated between gestations days 1-7. The decreases in absolute weight of the testis of F_1 males from treated rats between gestation days 7-14, 14-21, and 1-21 were not statistically significant. Absolute epididymal and prostate weight also decreased in F_1male rats from gestation days 1-7, 7- 14 and 1-21. F_1 male rats from gestation days 14-21 treated rats however increased but not significantly in absolute weight of the epididymis and prostate.

There were significant decreases ($p<0.05$) in the absolute liver weight in F_1 males from rats treated at gestation days 1-7 & 7-14 (Table 4.16). All F1 males from treated groups decreased in the absolute seminal vesicle weight, except gestation days 1-21 F_1 males that showed slight increase in absolute weight of the seminal vesicle. Absolute pituitary gland weight was significantly reduced ($p<0.05$) in F_1 males from gestation days 1-7 and 14-21 treated rats (Table 4.16)

Table 4.16: Absolute organ weight in adult F_1 males, from rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

Group by gestation period.n=5	Testes	Epididymis	Prostate	Liver	Sem.Vescle	Pit.gland
CONTROL	1.33±0.05	0.19±0.05	0.26±0.04	5.70±0.14	0.92±0.20	0.24±0.02
GD 1-7	0.99±0.04*	0.12±0.02	0.21±0.03	4.11±0.09**	0.65±0.09	0.11±0.02*
GD 7-14	1.00±0.19	0.11±0.03	0.16±0.04	4.77±0.28*	0.55±0.15	0.19±0.03
GD 14-21	1.28±0.02	0.21±0.02	0.27±0.04	5.71±0.28	0.69±0.10	0.11±0.01**
GD 1-21	1.31±0.03	0.16±0.02	0.23±0.03	5.65±0.29	1.00±0.09	0.30±0.06

KEY: * = p< 0.05, ** =p< 0.01

4.31 Effects of maternal exposure to *Ricinus communis* oil on relative organs weight in adult F_1 male

Relative weights of the testis decreased but not significantly in F_1 males from rats treated between gestation days 1-7 and 7-14. Relative epididymal weights decreased in F_1 males from rats treated between gestation days 1-7, 7-14 and 1-21, but increased in males from rats treated between gestation days 14-21. All changes were however not statistically significant. Relative weights of the prostate in F_1 males from all the treated rats were not significantly different from the control. The relative decrease in liver weights in F_1 males from gestation days 1-7 and 7-14 and the increase in F_1 males from gestation days 14-21 and 1-21 treated rats were not significantly different from control (Table 4.17). There were significant decreases ($p<0.05$), in the relative pituitary gland weight in F_1 males from gestation days 1-7 and 14-21 (Table 4.17).

Table 4.17: Percentage relative weight in adult F_1 males, from rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

Group by gestation day(GD) (N=5)	Testes (g)	Epididymis (g)	Prostate (g)	Liver (g)	Sem.Vescle (g)	Pit.gland (g)
CONTROL	0.57±0.02	0.08±0.02	0.11±0.02	2.44±0.07	0.39±0.08	0.10±0.01
GD 1-7	0.54±0.01	0.07±0.00	0.11±0.02	2.27±0.09	0.35±0.04	0.06±0.01*
GD 7-14	0.50±0.09	0.06±0.02	0.08±0.02	2.45±0.09	0.28±0.08	0.09±0.01
GD 14-21	0.57±0.01	0.09±0.01	0.12±0.01	2.56±0.11	0.31±0.04	0.05±0.00**
GD 1-21	0.58±0.01	0.07±0.01	0.10±0.01	2.52±0.05	0.45±0.04	0.13±0.04

KEY: * = p< 0.05, ** =p< 0.01

4.32 Effects of maternal exposure to *Ricinus communis* oil on epididymal sperm characteristics in adult F_1 male (sperm count, motility, viability, volume and percentage abnormal sperms)

Epididymal sperm count were significantly reduced ($p<0.01$) in all F_1 males from female rats treated with *Ricinus communis* oil at gestation days 1-7,7-14,14-21 and 1-21(Fig. 4.14). As shown in Figure 4.15 percentage epididymal sperm motility were significantly reduced ($p<0.05$) when compared with the control in F_1 males from female rats treated between gestation days 1-7, 7-14, 14-21 and 1-21. Similarly, as shown in Figure 4.16 and 4.17, epididymal sperm viability and volume reduced in all F_1 males from female rats treated at gestation days 1-7, 7-14, 14-21 and 1-21, the reductions were however not significantly different from the control. Figure 4.18 also showed that there were significant increases ($p<0.05$) in the percentage of abnormal sperms when compared with the control in all F_1 males from female rats treated at gestation days 1-7, 7-14, 14-21 and 1-21.

4.33 EPIDIDYMAL SPERM CHARACTERISTICS IN ADULT F_1 MALES FROM FEMALE RATS TREATED WITH *RICINUS COMMUNIS* OIL AT DIFFERENT GESTATION PERIODS (PND 90)

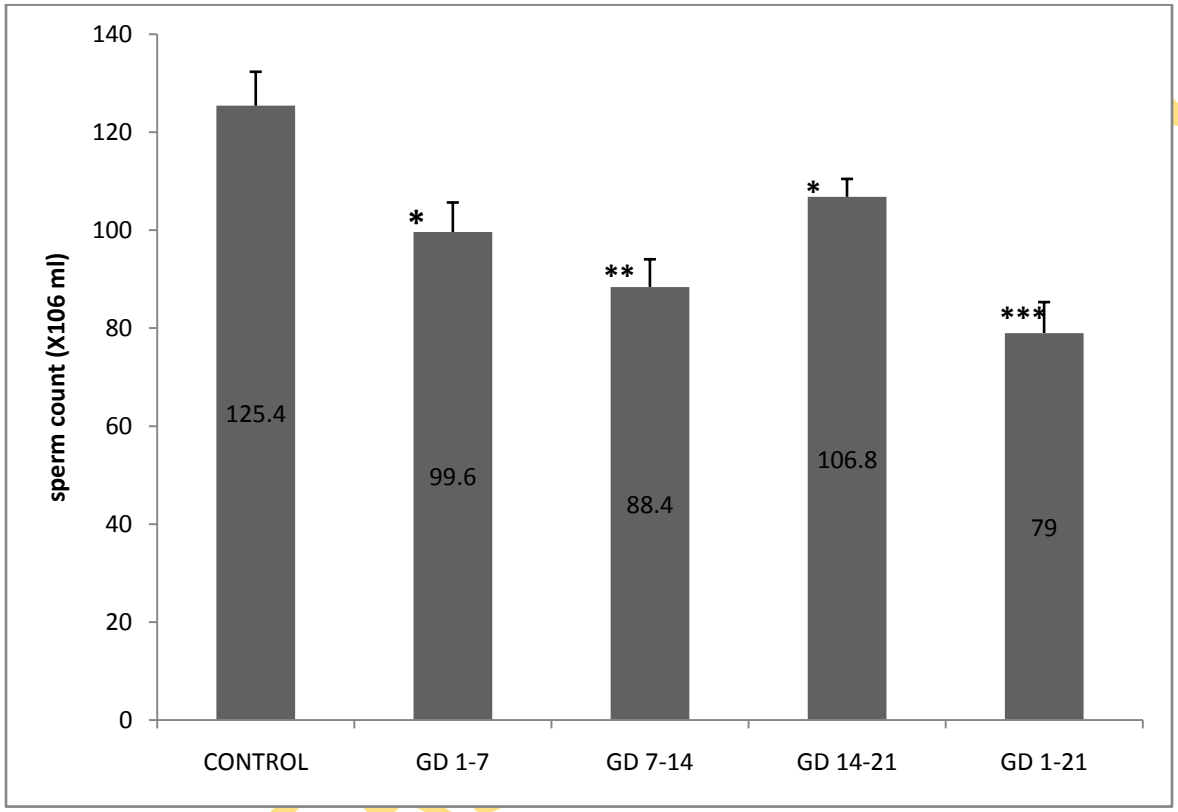

Figure 4.14: Epididymal sperm count in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: * = p< 0.05, ** =p< 0.01, *** =p< 0.001

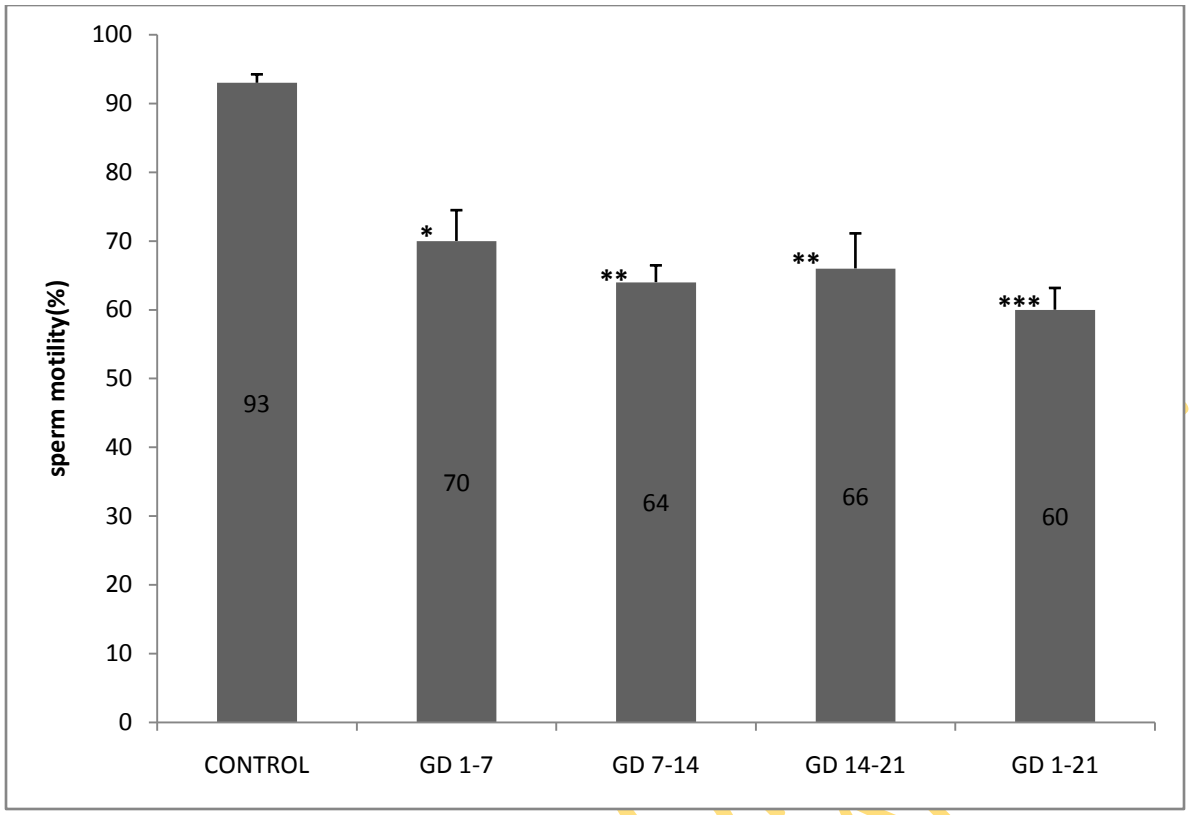

Figure 4.15: Percentage epididymal sperm motility in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: * = p< 0.05, ** =p< 0.01, *** =p< 0.001

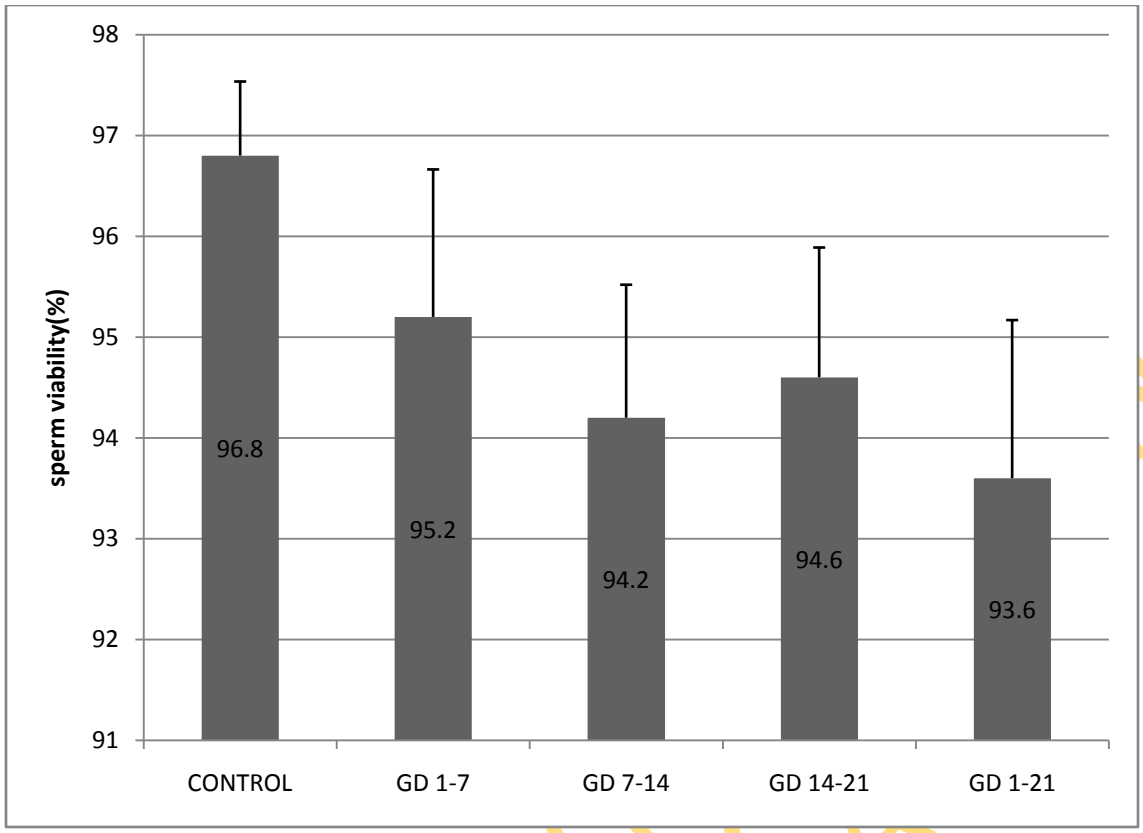

Figure 4.16: Epididymal sperm viability in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

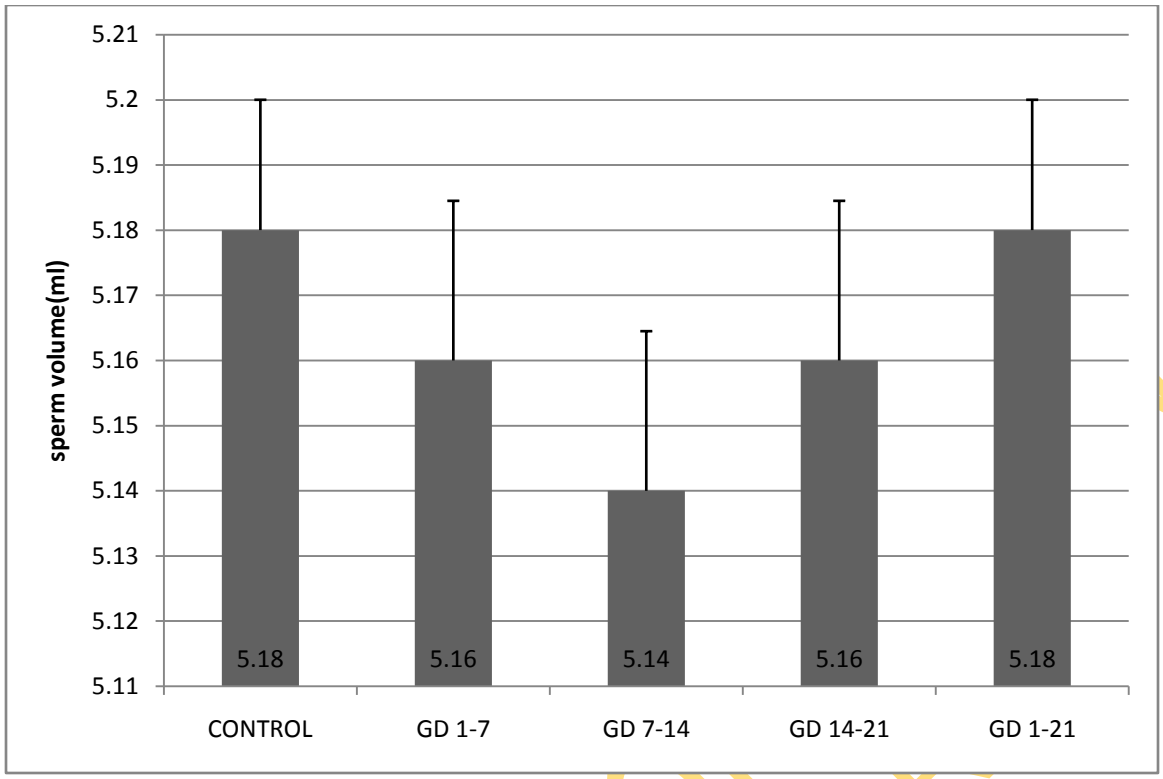

Figure 4.17: Epididymal sperm volume in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

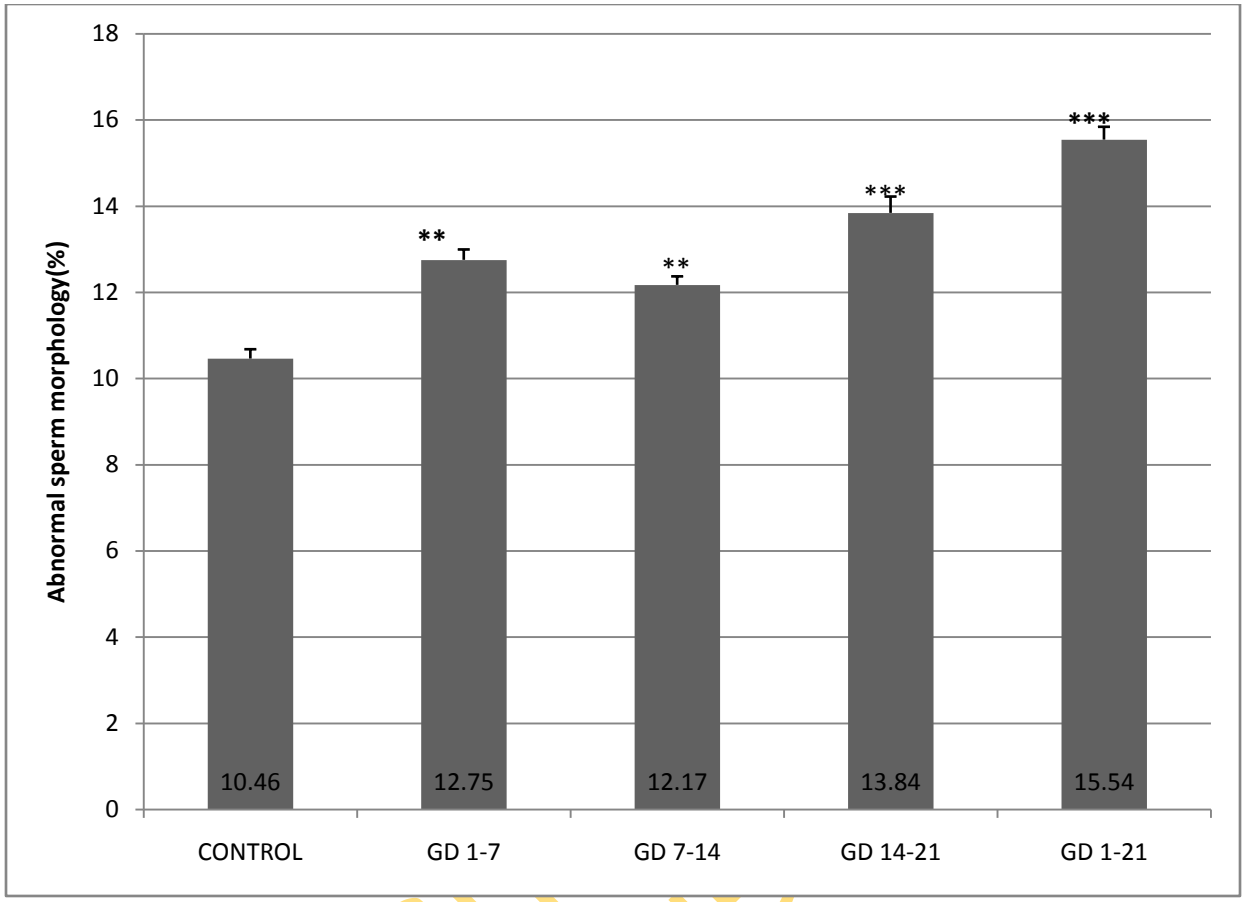

Figure 4.18: Percentage morphological aberrations in epididymal sperm in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: ** =p< 0.01, *** =p< 0.001

4.34 Effects of maternal exposure to *Ricinus communis* oil on serum FSH, LH, estrogen and testosterone in adult F_1 male

As shown in Figure 4.19 and 4.20, serum luteinizing hormone and follicle stimulating hormone levels were statistically increased ($p<0.05$) when compared with control in all F_1 males from *Ricinus communis* oil treated female rats. Serum estrogen level was also significantly increased (Figure 4.21) in F_1 males from gestation days 7-14 treated female rats while increases in F_1 males from gestation days 1-7 , 7-14 and 1-21 (Figure 4.21) were not statistically significant. However, as shown in Figure 4.22, serum testosterone levels significantly decreased ($p<0.01$) when compared with control in F_1 males from gestation days 1-7, 7-14, 14-21 and 1-21.

4.35 HORMONE PROFILE IN ADULT F_1 MALES FROM FEMALE RATS TREATED WITH *RICINUS COMMUNIS* OIL AT DIFFERENT GESTATION PERIODS (PND 90)

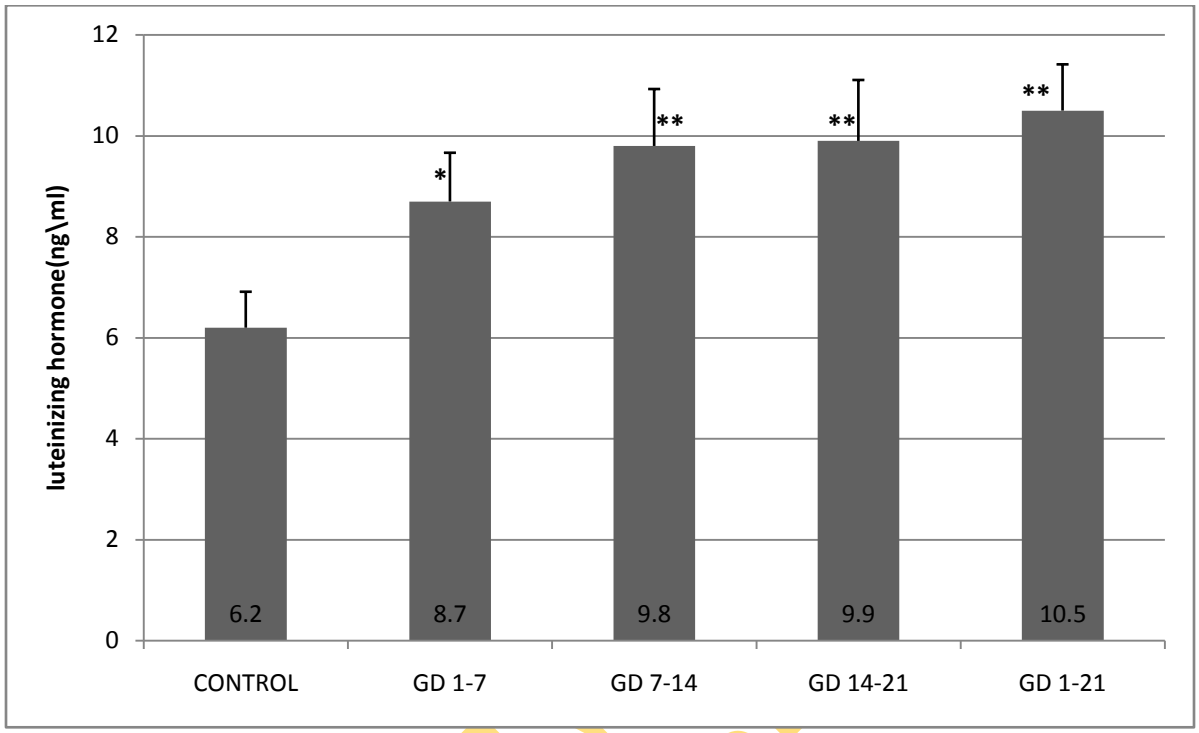

Figure 4.19: Serum Luteinizing hormone level in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: * = p< 0.05, ** =p< 0.01

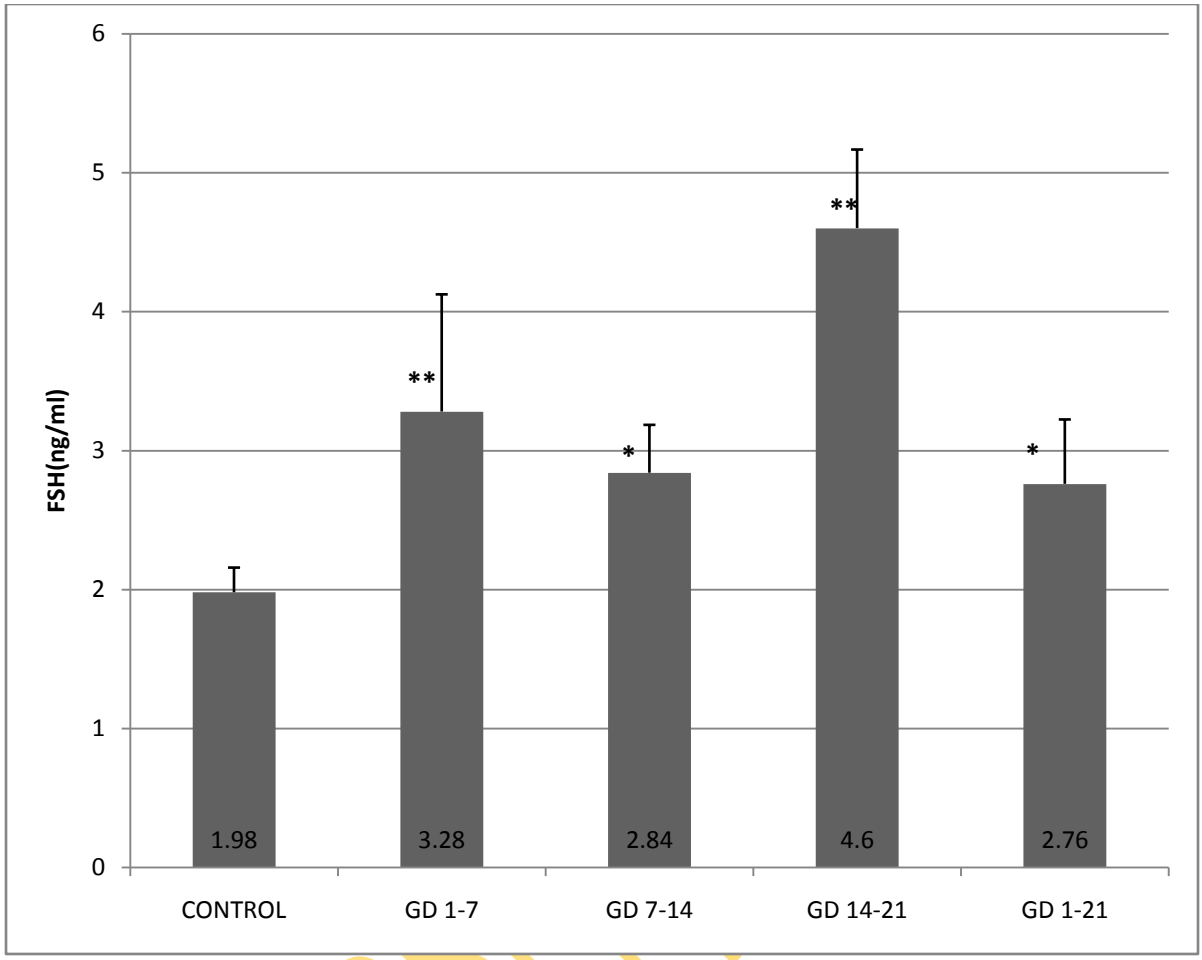

Figure 4.20: Serum follicle stimulating hormone (FSH) level in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: * = p< 0.05, ** =p< 0.01

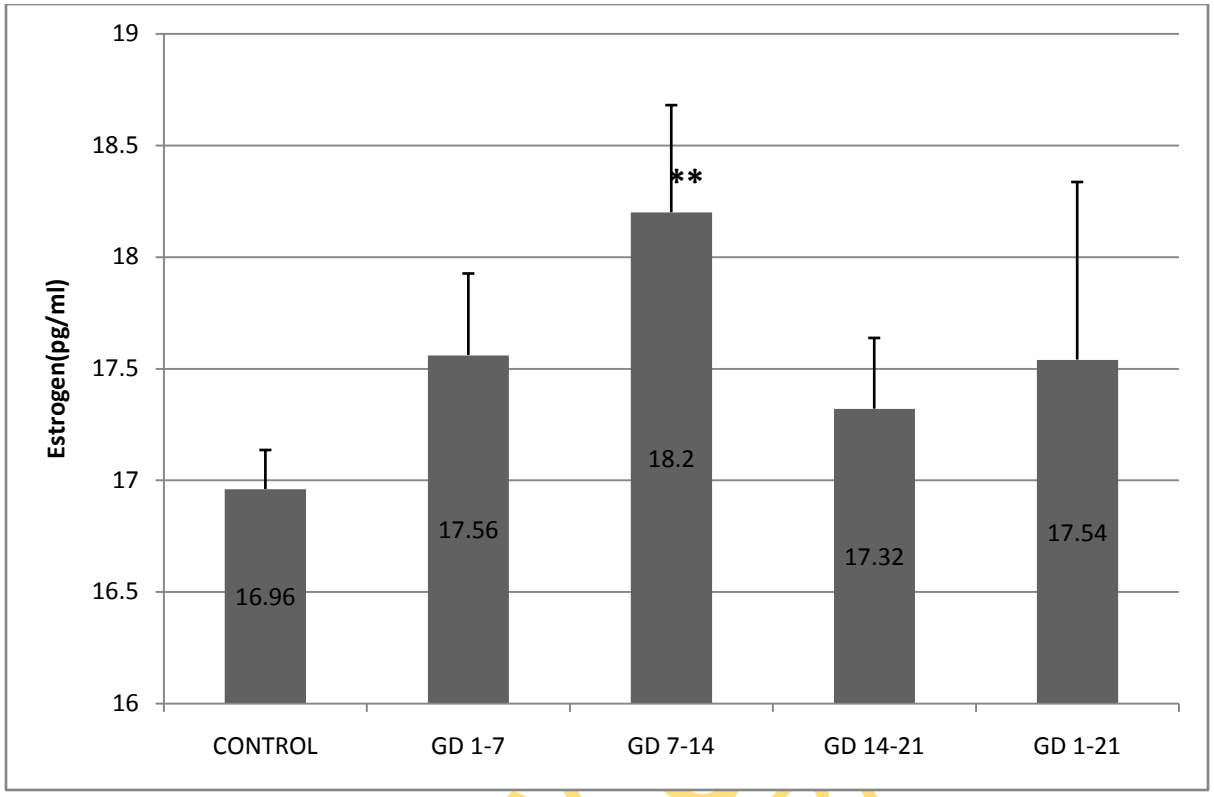

Figure 4.21: Serum estrogen level in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: a = p< 0.05, b =p< 0.01, c =p< 0.001

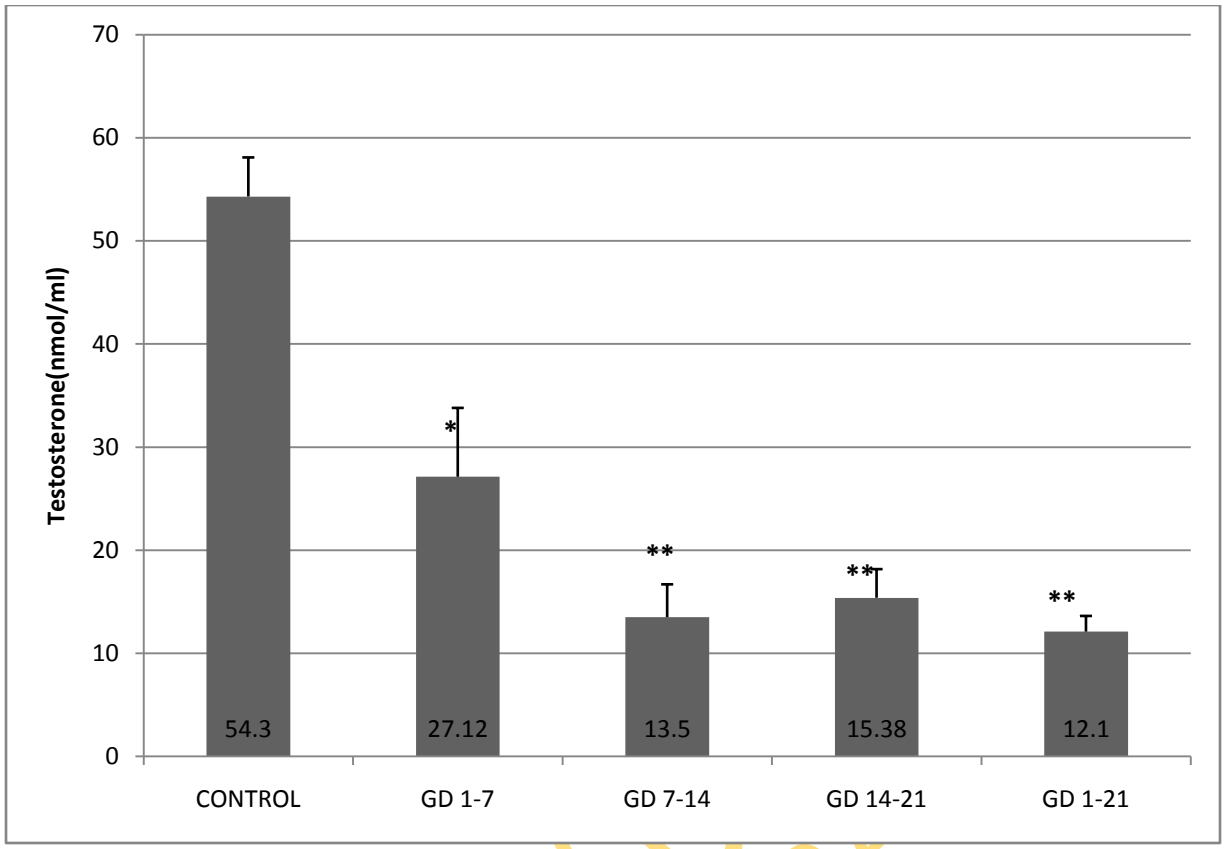

Figure 4.22: Serum testosterone level in adult F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods (PND 90)

KEY: * = p< 0.05, ** =p< 0.01

4.36 Effects of maternal exposure to *Ricinus communis* oil on histology of the testis and epididymis in adult F_1 male

There were no visible lesions in the photomicrographs of both the testis and epididymis of F_1 males from the control group (Plates 4.11a and b, Plates 4.16a and b). Plates 4.12a and b of the testis of F_1 adult males from gestation days 1-7 treated rats at sacrifice on PND 90 showed mild interstitial oedema; however there were no visible lesions in the epididymis (Plates 4.17a and b). Plates 4.13a, b and c of the testis of adult F_1 males from gestation days 7-14 treated rats showed mild interstitial oedema with reduced seminiferous tubular lumen. Photomicrograph of the epididymis of F_1 adult male (plate 4.18) from this group was normal except for sparse luminal content. Photomicrographs of the testis of adult F_1 male from gestation days 14-21 (plate 4.14) showed mild interstitial oedema while the epididymis of F_1 male from same group (plate 4.19) showed no lesions but reduced luminal content. Plate 4.15 showed the photomicrograph of the testis of F_1 male from gestation days 1-21 showing severe subcapsular congestion while the epididymis (plate 4.20) showed no visible lesions.

4.37 Photomicrographs of the testis and epididymis of F_1 males from female rats treated with *Ricinus communis* oil at different gestation periods.

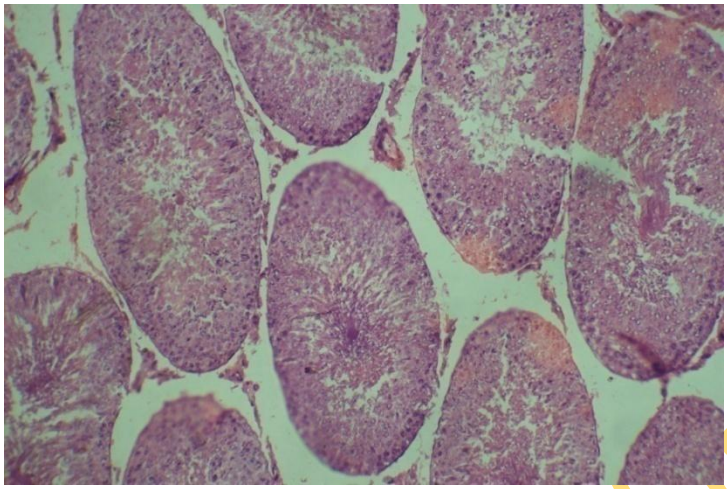

Plate 4.11a: Photomicrograph of the testis of F_1 male from control group (X200)

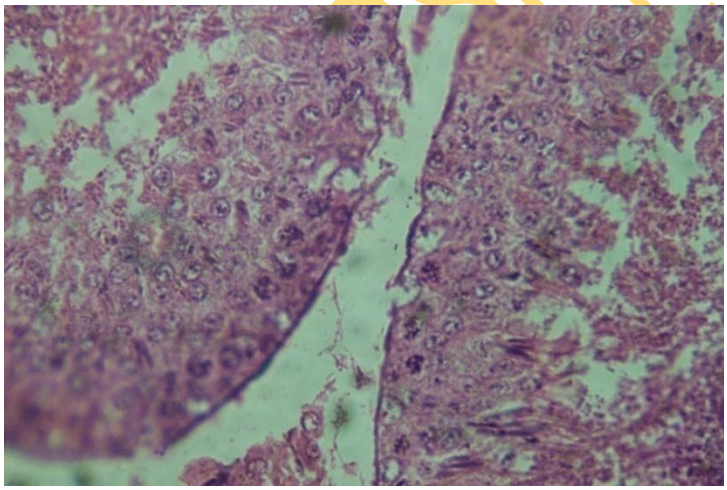

Plate 4.11b: Photomicrograph of the testis of F_1 male from control group(X400)

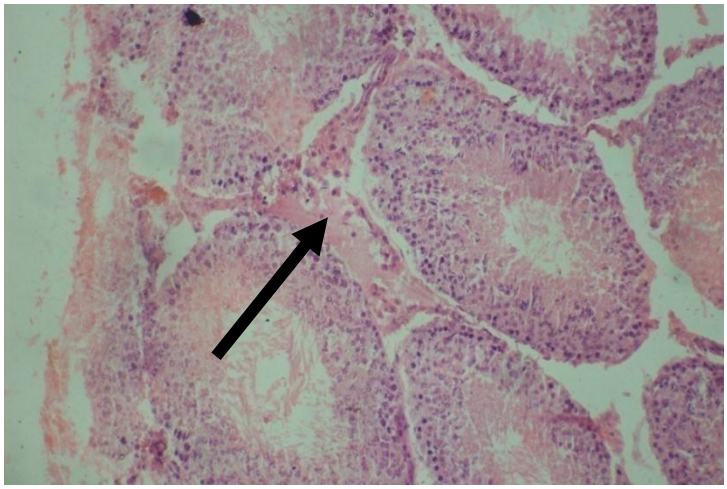

Plate 4.12a: Photomicrograph of the Testis of adult F_1 male from gestation days 1-7 treated rats showing slight interstitial oedema (X200)

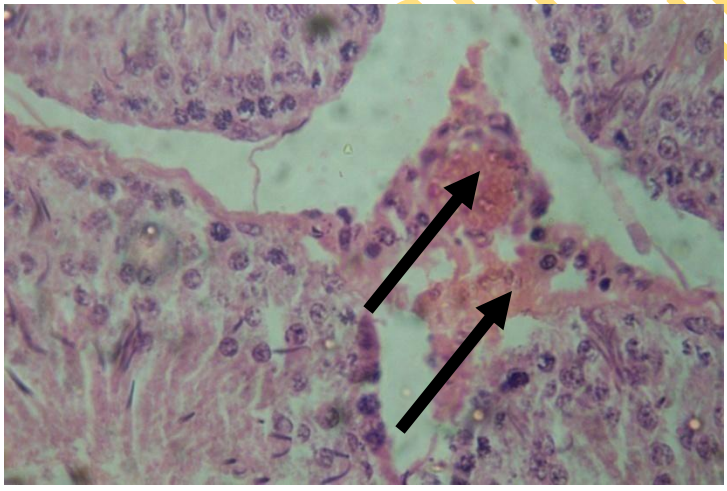

Plate 4.12b: Photomicrograph of the Testis of adult F_1 male from gestation days 1-7 treated rats, showing slight interstitial oedema (X400).

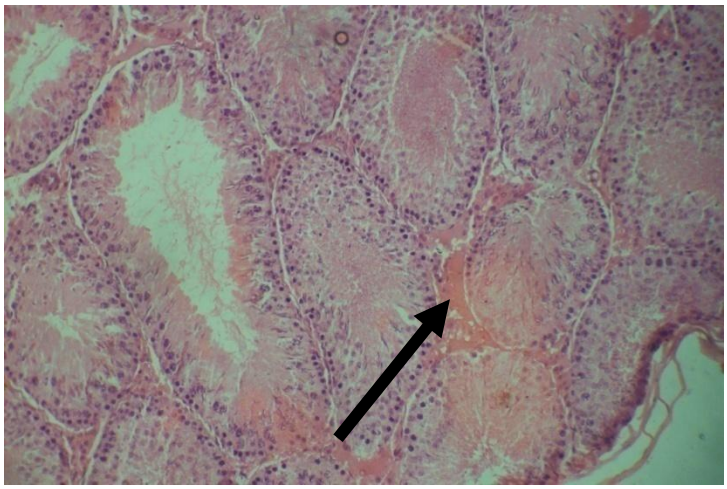

Plate 4.13a: photomicrograph of the testis of adult F_1 male from GD 7-14 showing mild interstial oedema (X200)

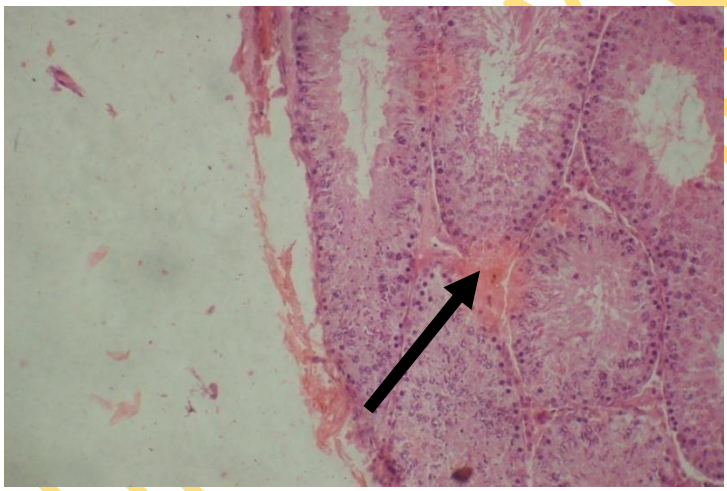

Plate 4.13b: photomicrograph of the testis of adult F_1 male from GD 7-14 showing mild interstial oedema(X400)

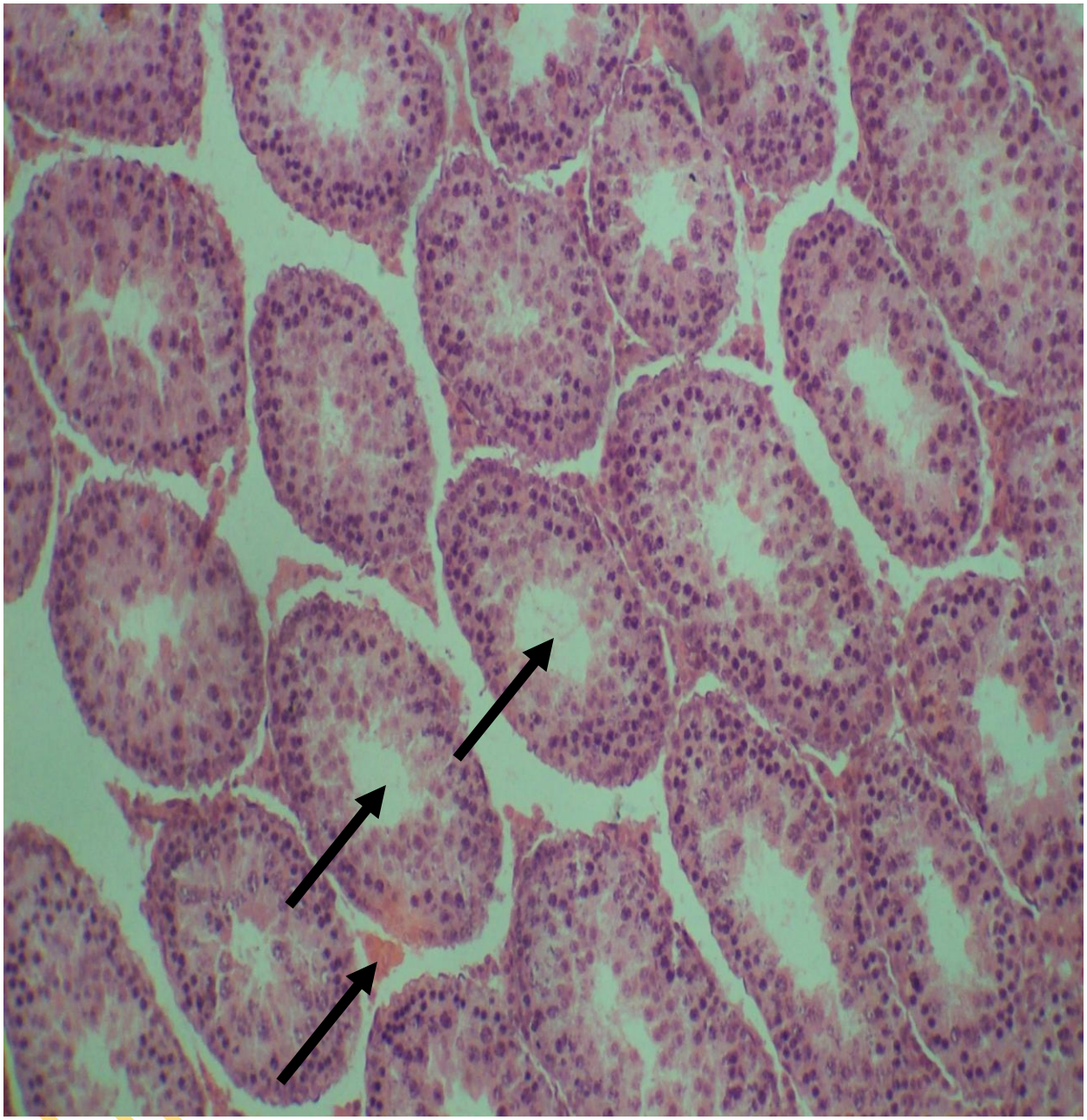

Plate 4.13c: photomicrograph of adult F_1 male from GD 7-14 showing reduced seminiferous tubular lumen diameter and interstitial oedema (X200)

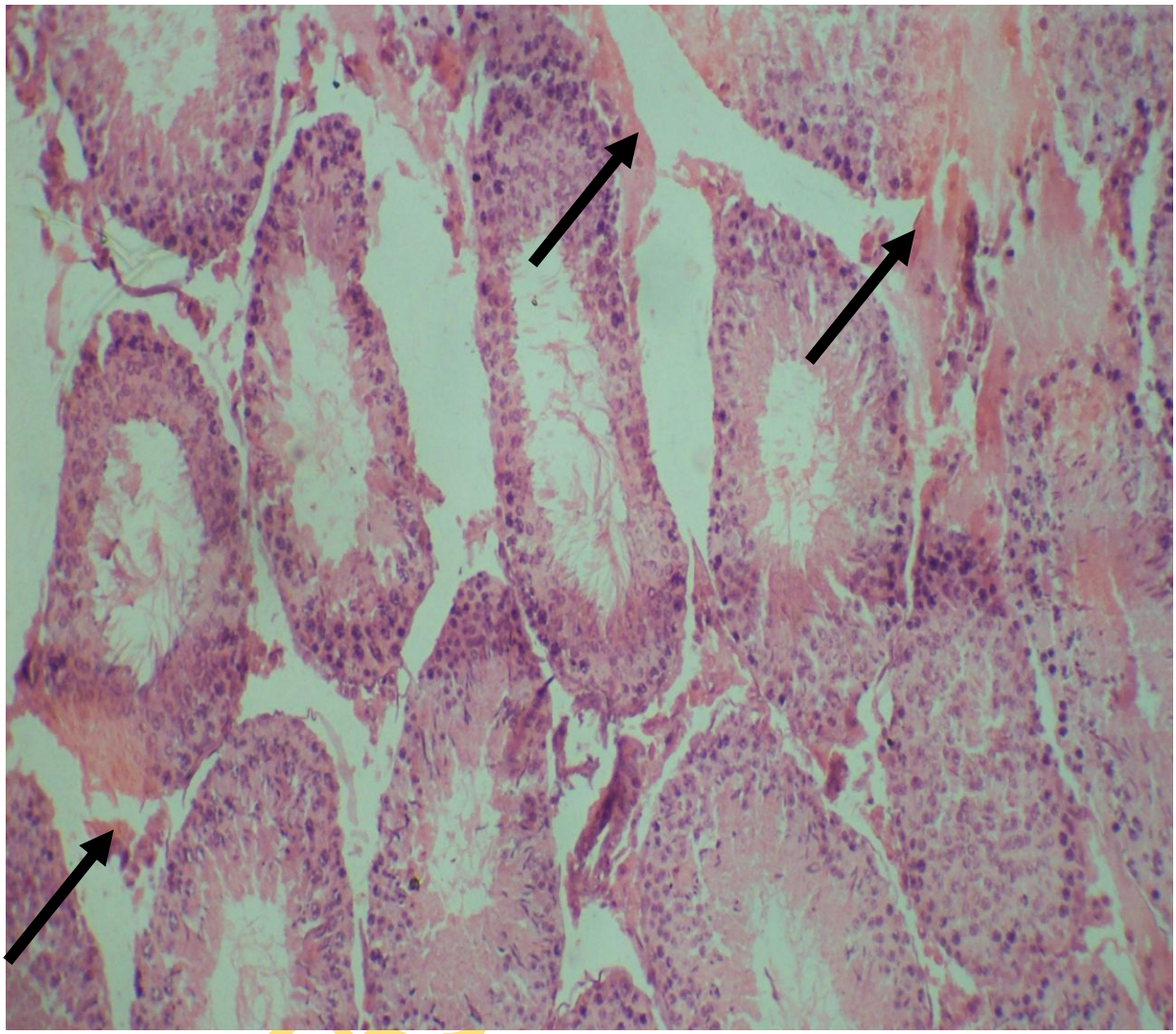

Plate 4.14: Photomicrograph of the testis of adult F_1males from gestation days 14- 21 treated rats showing slight interstitial oedema (X200)

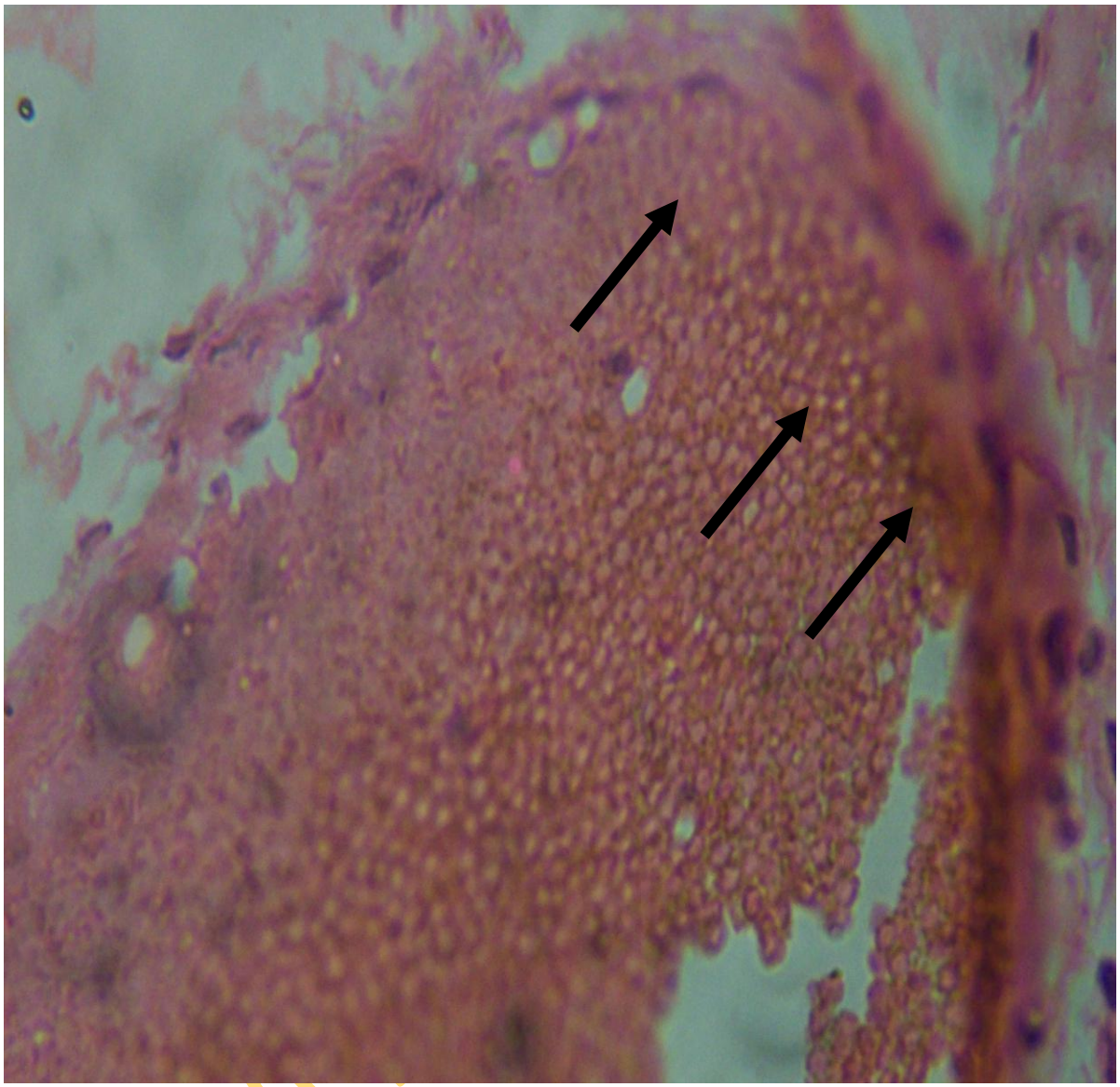

Plate 4.15: Photomicrograph of the testis from adult F_1 males from gestation days 1-21 treated rats showing severe subcapsular congestion. (X400).

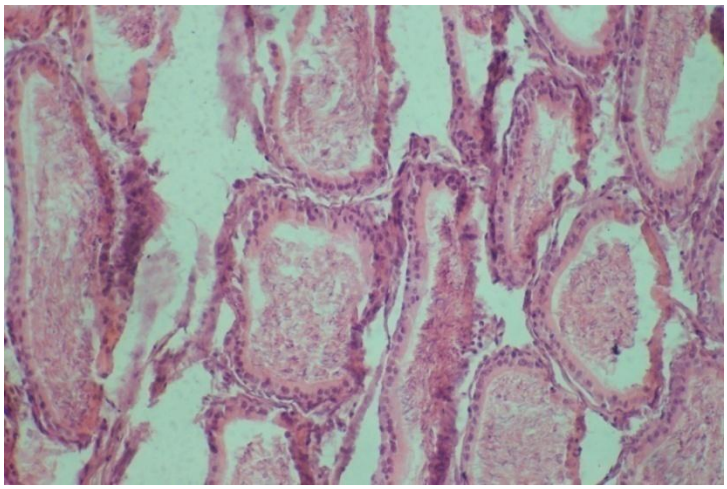

Plate 4.16a: Photomicrograph of the epididymis from control F_1 adult male rat(X200)

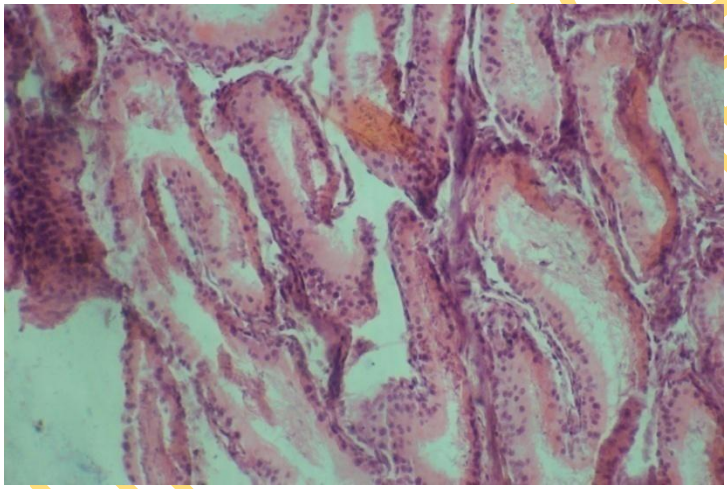

Plate 4.16b: Photomicrograph of the epididymis from control F_1 adult male rat(X200)

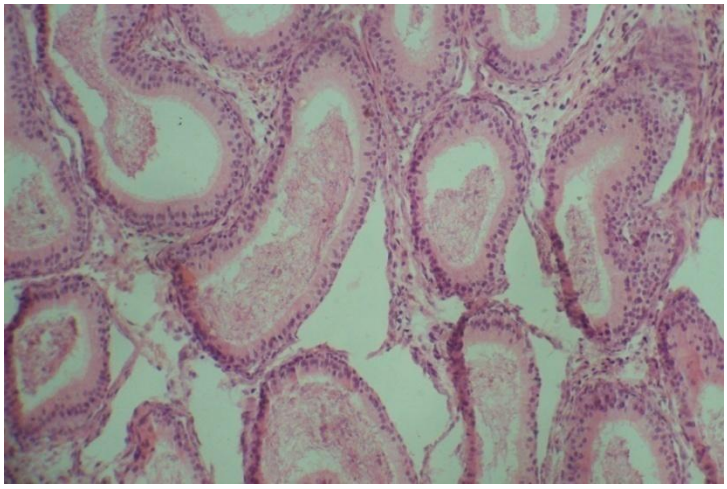

Plate 4.17a: Photomicrograph of the epididymis of adult F_1 male from gestation days 1-7 treated rats showing no visible lesions (X200)

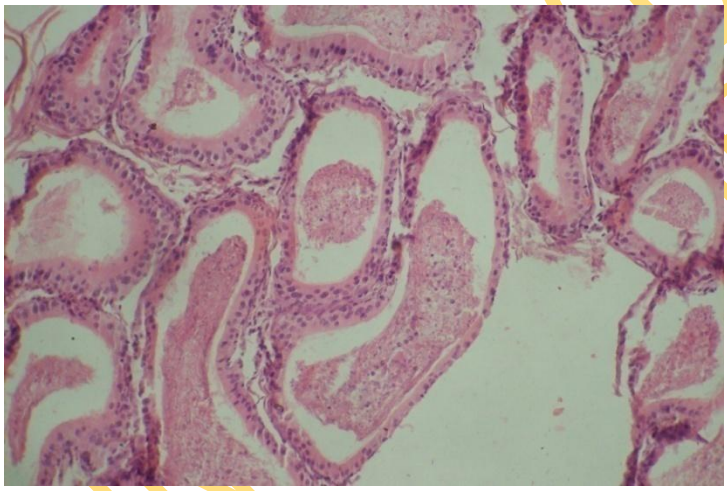

Plate 4.17b: Photomicrograph of the epididymis of adult F_1 male from gestation days 1-7 treated rats showing no visible lesions (X200)

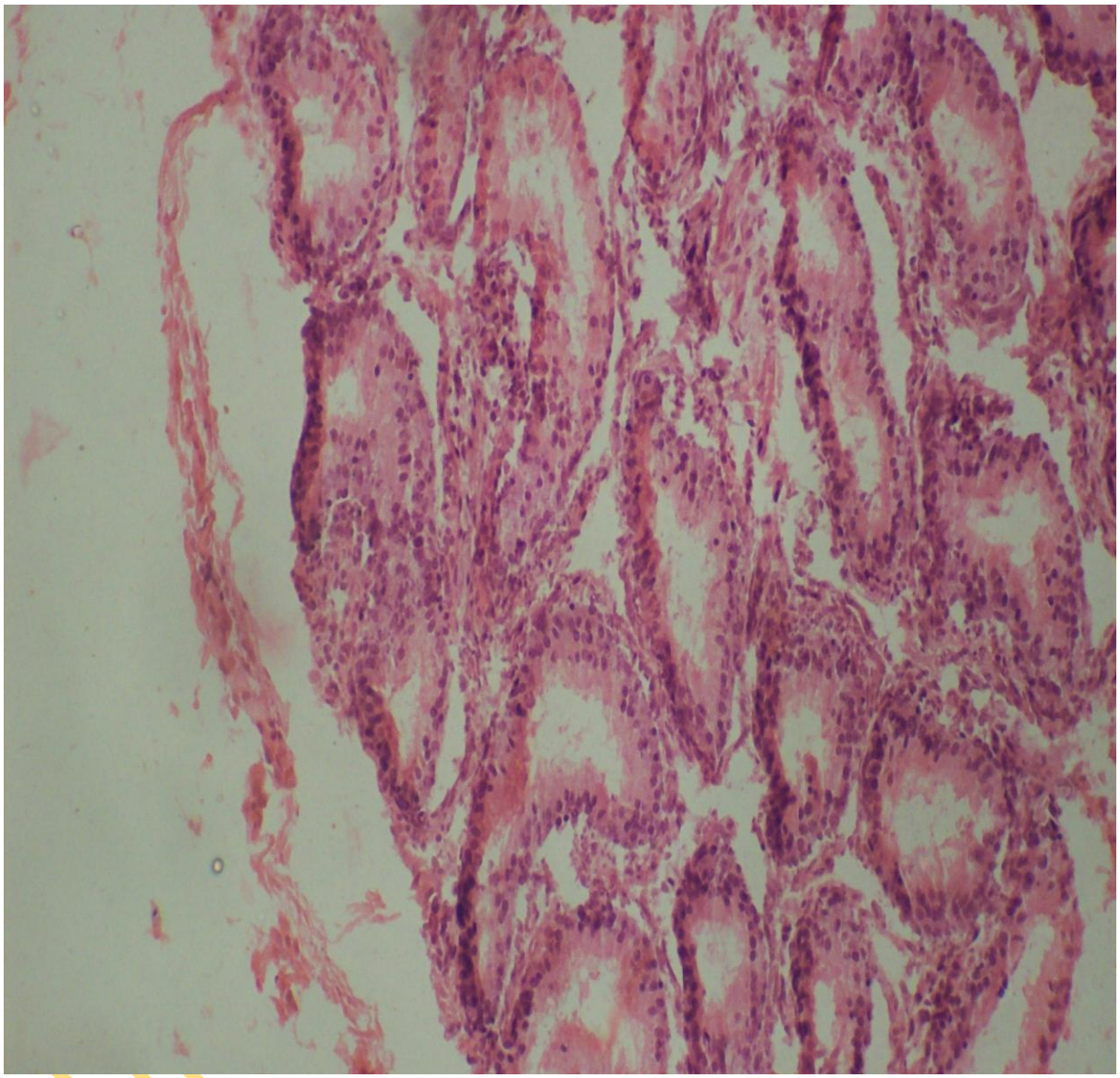

Plate 4.18: photomicrograph of adult F_1 male epididymis from GD 7-14 showing sparse luminal content(X200)

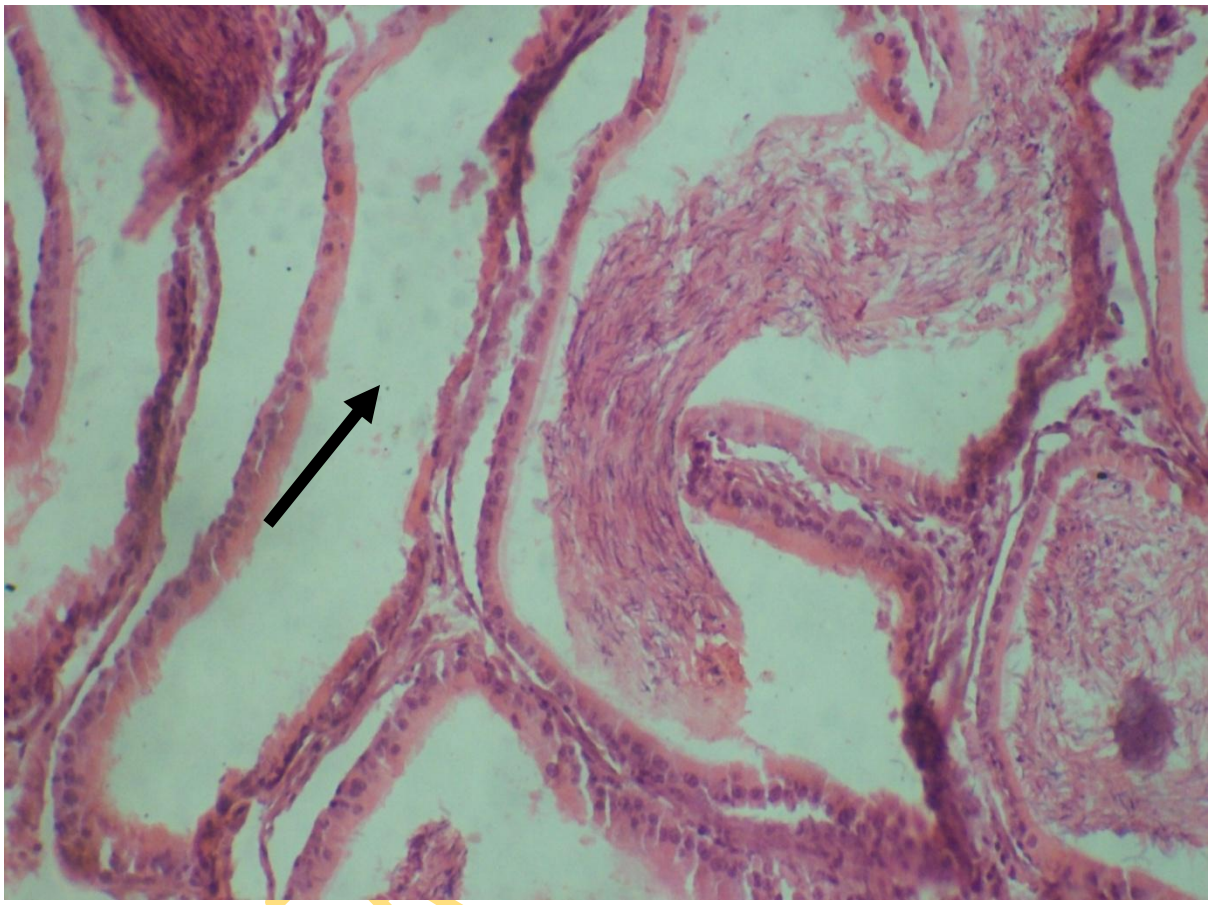

Plate 4.19: Photomicrogrph of the epididymis in F_1 adult male from gestation days 14-21 treated rats showing no visible lesion but sparse luminal content (X200)

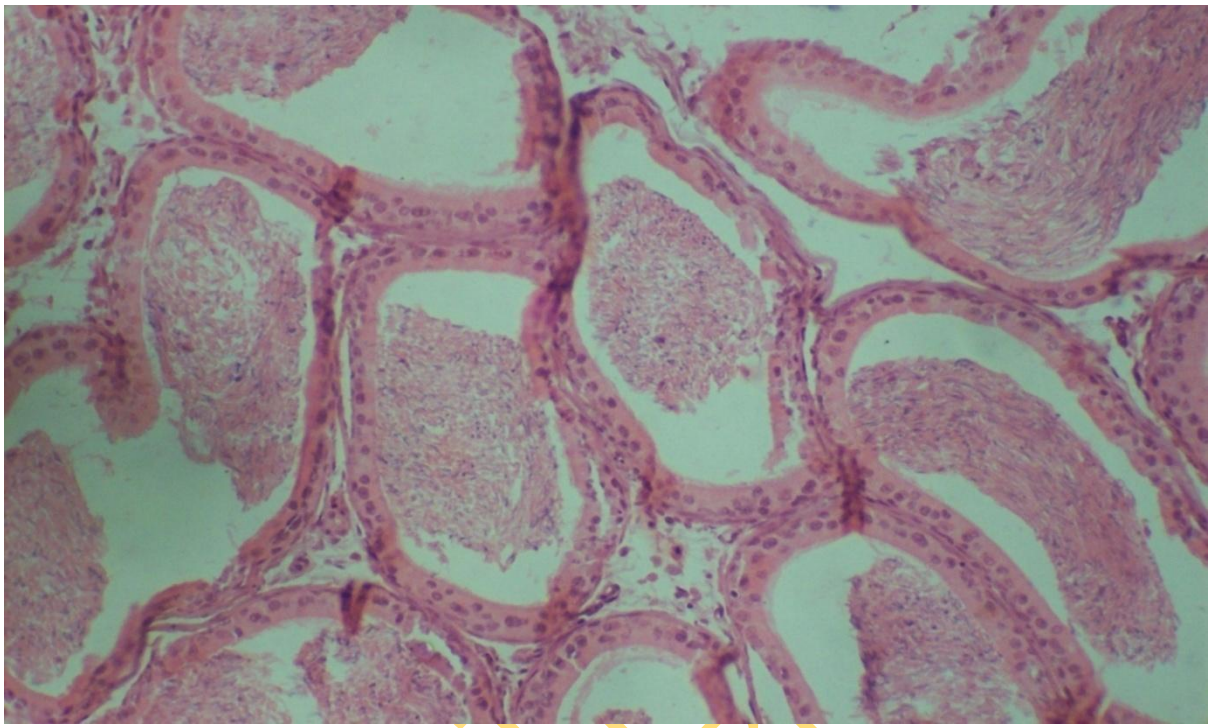

Plate 4.20: Photomicrograph of the epididymis of adult F_1 males from GD days 1-21 treated rats without visible lesions seen.

4.38 Effects of maternal exposure to *Ricinus communis* oil on histology of ovary and uterus in adult F_1 female

As shown in plates 4.21a/ b, 4.22a/ b and 4.23a/b, no visible lesions were seen in the ovaries of F_1 females from gestation days 1-7, 14-21 and 1-21 treated rats. Inaddition, follicles at different stages of development can be seen in the ovaries.

As shown in Plates 4.24a and b, there was tissue disruption in the uterus of F1 adult female from GD 1-7. However, no lesions were seen in the uterus of adult F_1 female from GD 1-21 (plates 4.27a and b). There was reduced uterine mucosa depth in adult F_1 female from GD 7-14 (plates 4.25a and b). Glandular features in the uterus of F_1 female from gestation days 14-21 were not prominent as shown in plates 4.26a and b).

4.39 Photomicrographs of the ovary and the uterus of F_1 females (PND 90) from rats treated with *Ricinus communis* oil at different gestation periods.

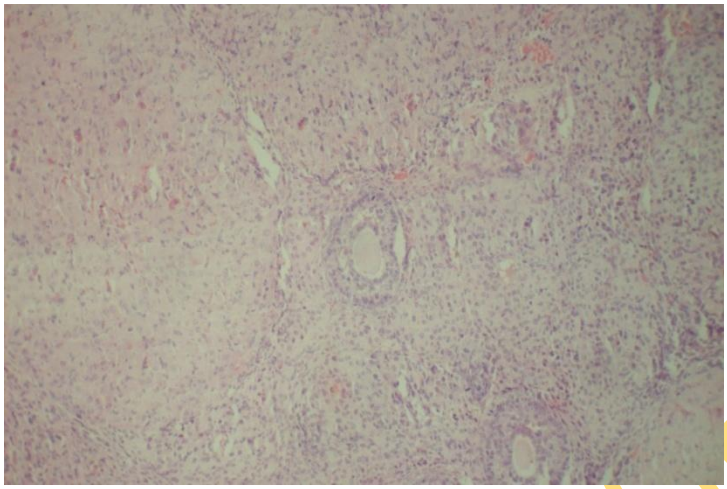

Plate 4.21a: Photomicrograph of the ovary of adult F_1 female from gestation days 1-7 treated rats (X200)

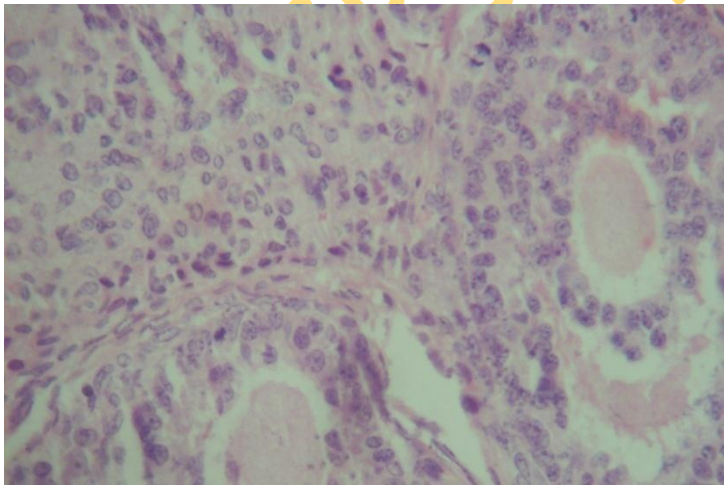

Plate 4.21b: Photomicrograph of the ovary of adult F_1 female from gestation days 1-7 treated rats showing no visible lesions (X400)

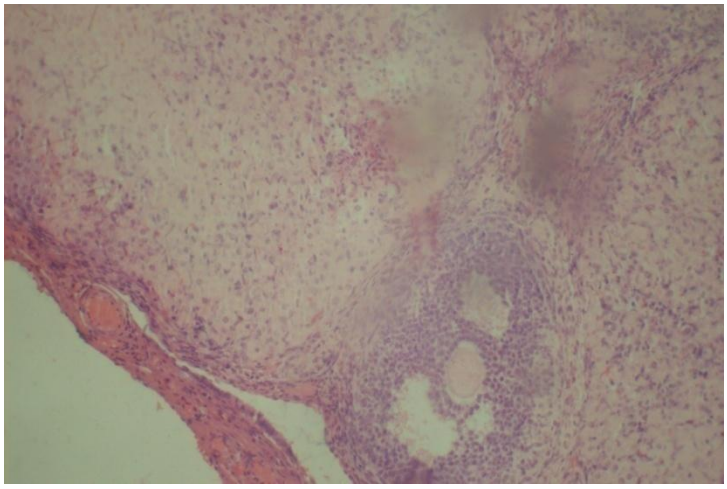

Plate 4.22a: Photomicrograph of the ovary in adult F_1 female from gestation days 14-21 treated rats showing no visible lesions seen(X200)

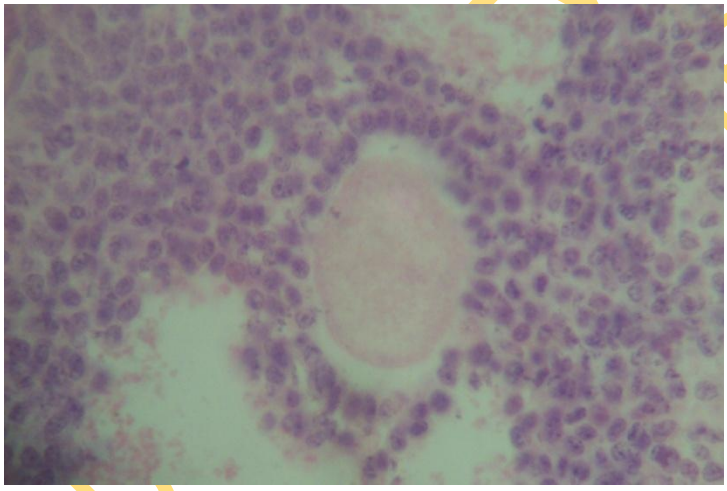

Plate 4.22b: Photomicrograph of the ovary in adult F_1 female from gestation days 14-21 treated rats showing no visible lesions seen (X400)

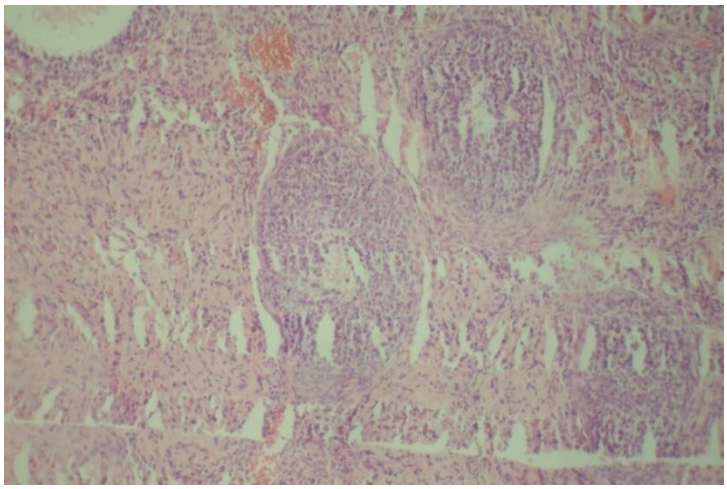

Plate 4.23a: Photomicrograph of the ovary of adult F_1 female from rats treated between gestation days 1-21 (X200)

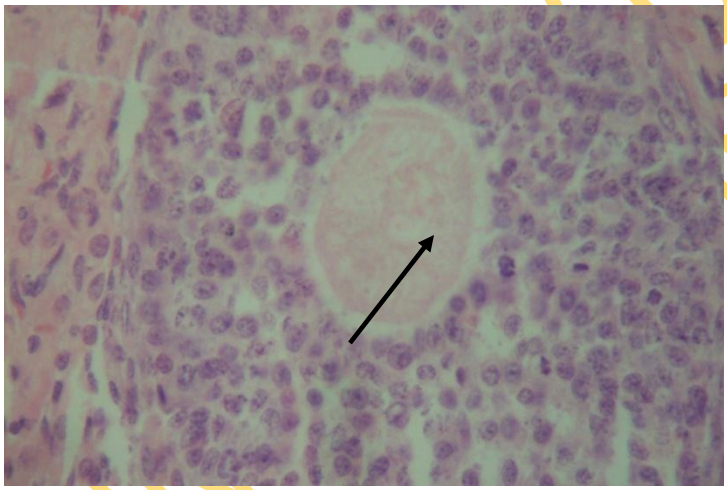

Plate 4.23b: Photomicrograph of the ovary of adult F_1 female from rats treated between gestation days 1-21 showing normal developing secondary follicles (X400)

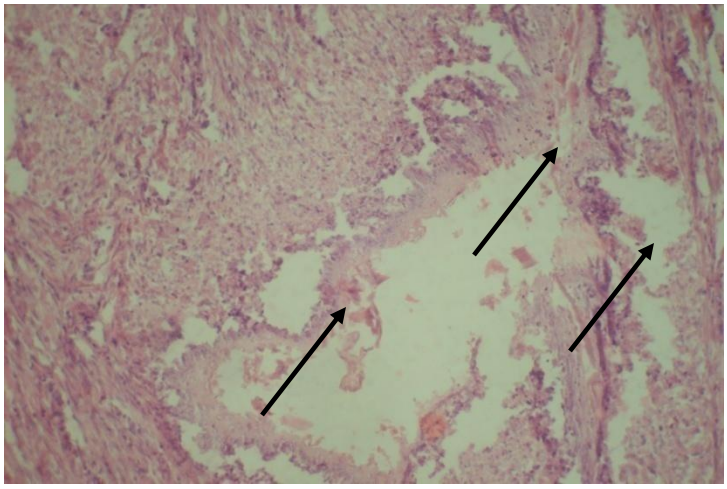

Plate 4.24a: Photomicrograph of the uterus of adult F_1 female from gestation days 1-7 treated rats showing tissue disruption (X200)

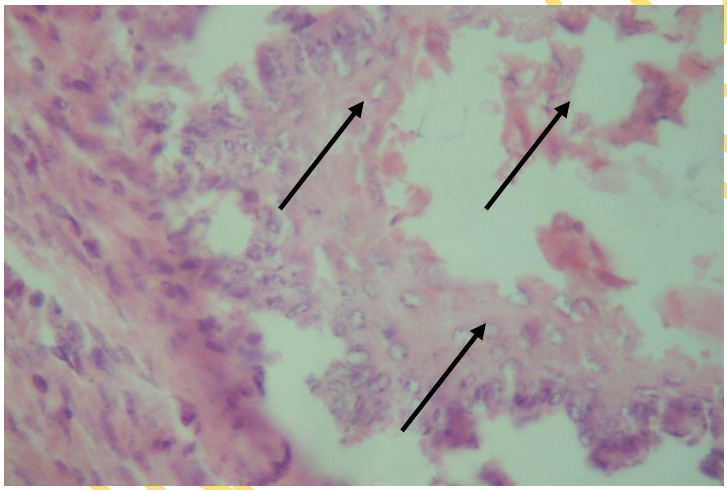

Plate 4.24b: Photomicrograph of the uterus of adult F_1 female from gestation days 1-7 treated rats showing no visible lesions, except minor tissue disruption (X400)

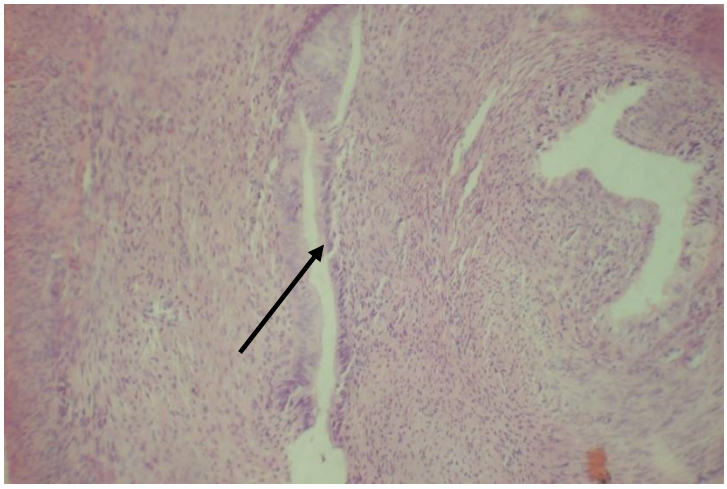

Plate 4.25a: Photomicrograph of the uterine tube of adult F_1 female from gestation days 7-14 treated rats showing slightly reduced mucosal depth (X200)

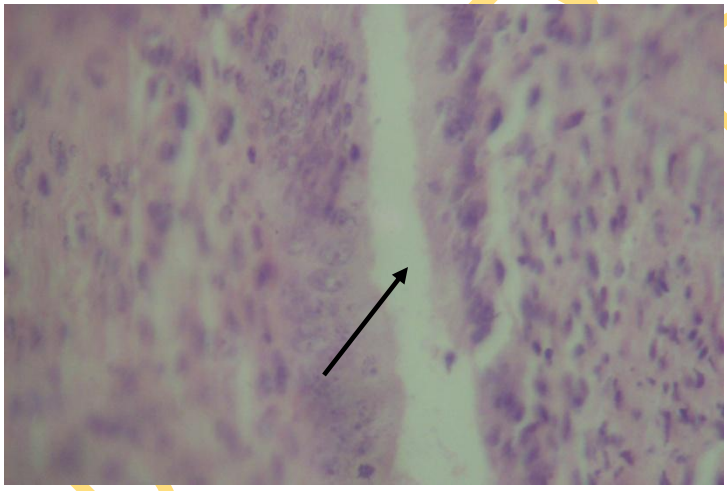

Plate 4.25b: Photomicrograph of the uterine tube of adult F_1 female from gestation days 7-14 treated rats showing slightly reduced mucosal depth (X400)

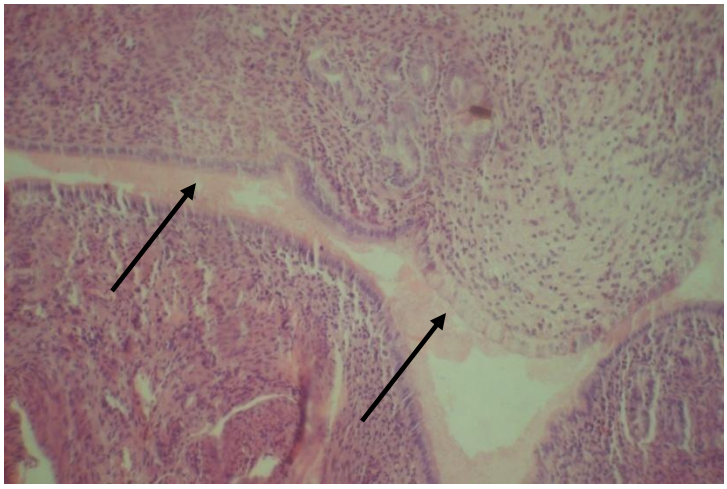

Plate 4.26a: Photomicrograph of the uterus in adult F_1 female from gestation days 14-21 treated rats showing glandular features that are not prominent and pink staining in the lumen(X200)

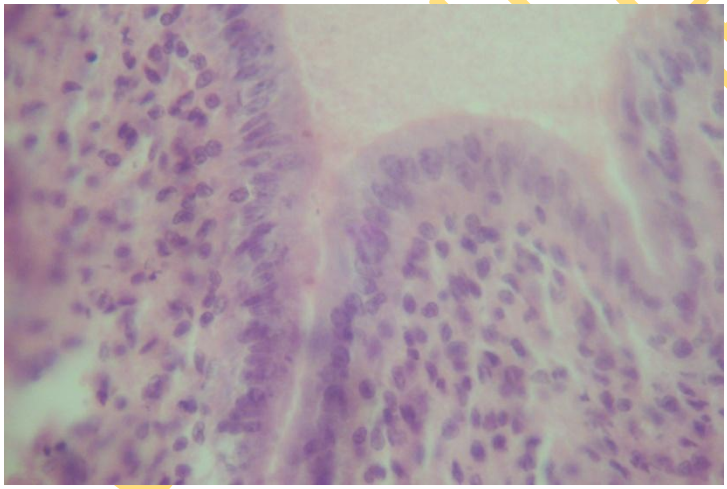

Plate 4.26b: Photomicrograph of the uterus in adult F_1 female from gestation days 14-21 treated rats showing glandular features that are not prominent and pink staining in the lumen(X400)

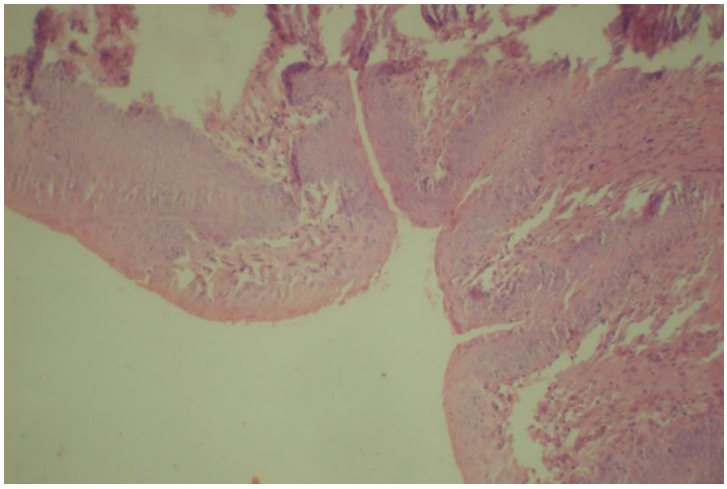

Plate 4.27a: Photomicrograph of the uterus in adult F_1 female from gestation days 1-21 treated rats showing no visible lesions seen (X200)

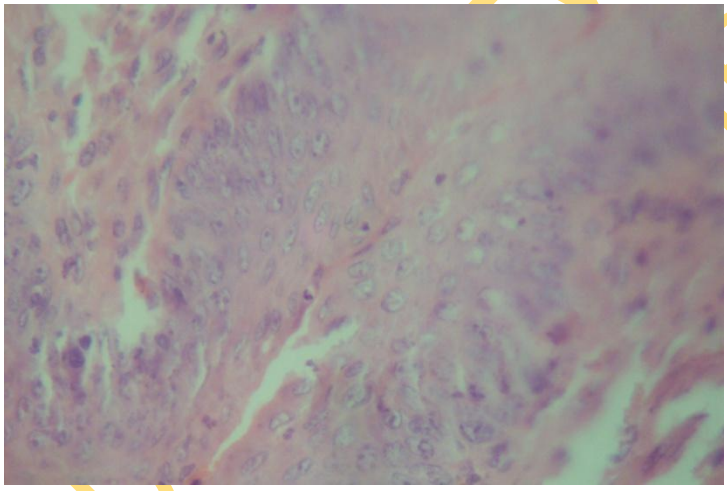

Plate 4.27b: Photomicrograph of the uterus in adult F_1 female from gestation days 1-21 treated rats showing no visible lesions seen (X400)

4.40 Effects of maternal exposure to *Ricinus communis* oil on reproductive indices in F_1 male and female

Mating index, fertility index, gestation index, life birth index, and day survival index were all 100% in control F_1 male and female mating experiments except lactation index with a percentage of 92.8% (Table 4.18). F_1 male and female from gestation days 1-7, 7-14 and 1-21 treated rats after mating experiments presented with a mating index of 100%; fertility, gestation, life birth, day survival and lactation indices were however all 0% (Table 4.18). F_1 male and female from gestation days 14-21 treated rats after mating experiment presented with 100% mating, fertility, gestation, and life birth indices. Day survival and lactation indices were however 89.5% (Table 4.18). F_1 male from control and gestation days 1-7, 7-14, 14-21 and 1-21 treated rats with female of untreated mothers showed 100% level in mating, fertility, gestation, life birth, day survival and lactation indices (Table 4.19).

4.41 Reproductive indices from mating experiments in F_1 male and female from mothers exposed to *Ricinus communis* oil

Table 4.18: Reproductive indices from mating experiment on male and female offspring (F_1) from treated mothers

PARAMETERS	**CONTROL**	**GD 1-7**	**GD 7-14**	**GD 14-21**	**GD 1-21**
MATING INDEX	100%	100%	100%	100%	100%
FERTILITY INDEX	100%	0%	0%	100%	0%
GESTATION INDEX	100%	0%	0%	100%	0%
LIVE BIRTH INDEX	100%	0%	0%	100%	0%
DAY SURVIVAL INDEX	100%	0%	0%	89.5%	0%
LACTATION INDEX	92.80%	0%	0%	89.5%	0%

Table 4.19: REPRODUCTIVE INDICES FROM MATING EXPERIMENT OF MALE OFFSPRING (F_1) FROM TREATED MOTHERS WITH UNTREATED FEMALE

PARAMETERS	**CONTROL**	**GD 1-7**	**GD 7-14**	**GD 14-21**	**GD 1-21**
MATING INDEX	100%	100%	100%	100%	100%
FERTILITY INDEX	100%	100%	100%	100%	100%
GESTATION INDEX	100%	100%	100%	100%	100%
LIVE BIRTH INDEX	100%	100%	100%	100%	100%
DAY SURVIVAL INDEX	100%	100%	100%	100%	100%
LACTATION INDEX	100%	100%	100%	100%	100%

4.42 Effects of maternal exposure to *Ricinus communis* oil on weekly weight until weaning in F_2 female and male pups.

As shown in Figure 4.23, there were significant decreases ($p<0.05$) in the body weight when compared with the control from the first to the third week of F_2 female from F_1 male and female from gestation days 14-21 treated rats. There were significant decreases ($p<0.05$) in the body weight when compared to the control at the second and third week of F_2 male from gestation days 14-21 treated rats. Week 1 weight of F_2 male also decreased but not significantly (Figure 4.24).

4.43 F_2 DATA FROM MATING EXPERIMENTS ON F_1 MALE AND FEMALE OFFSPRING WITH TREATED PARENTS

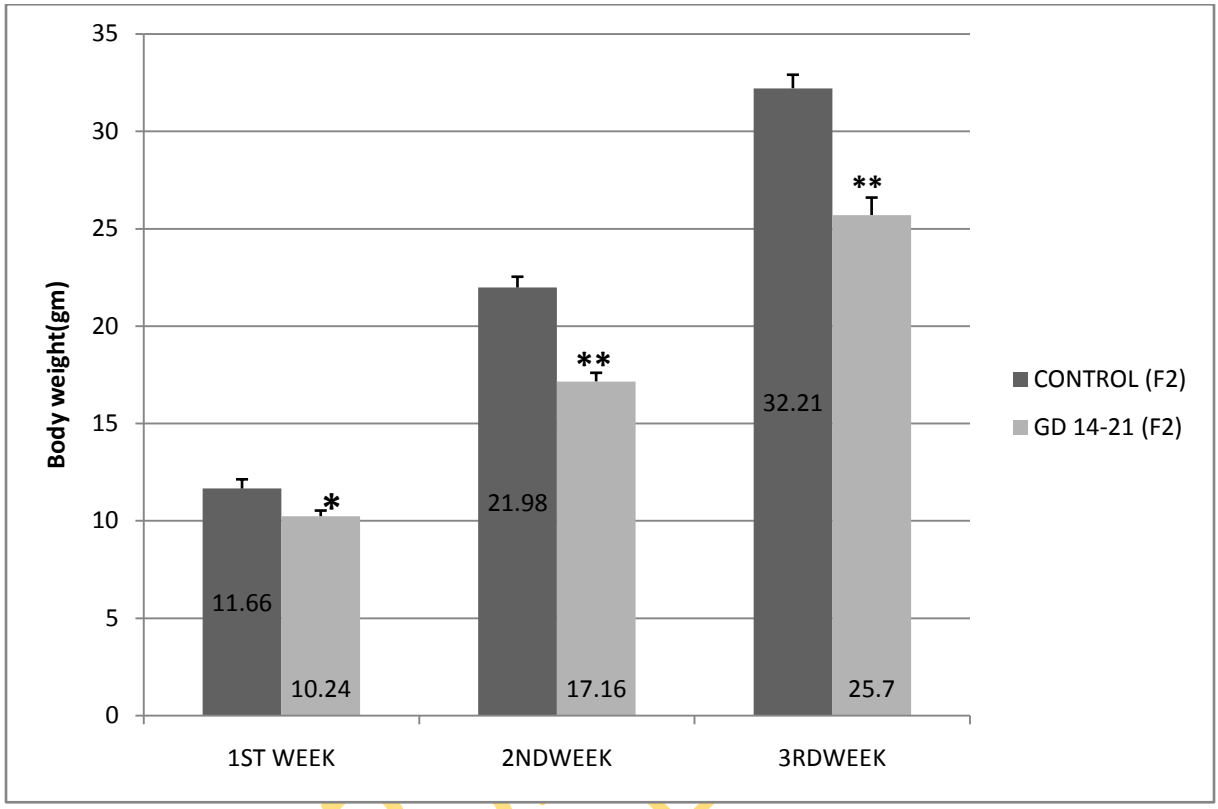

Figure 4.23: Weekly female F_2 pups weight until weaning at PND 21(third week)

KEY: * = p< 0.05, ** =p< 0.01

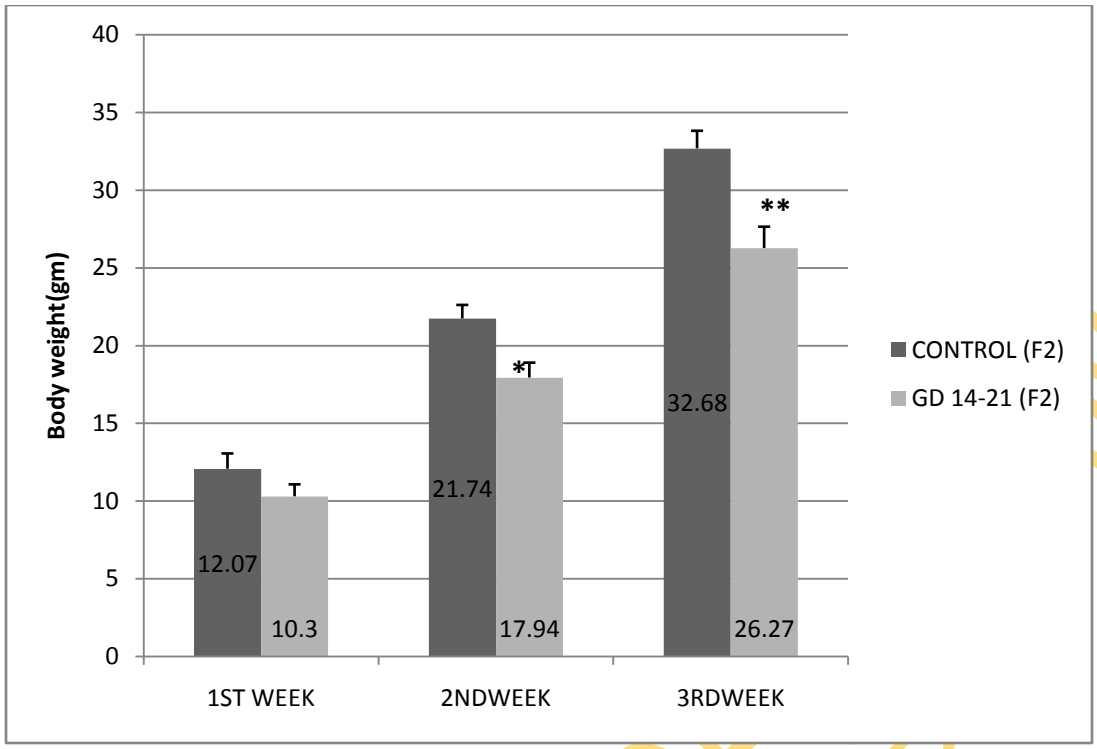

Figure 4.24: Weekly male F_2 pup weights until weaning at PND 21 (third week)

KEY: * = p< 0.05, ** =p< 0.01

4.44 Effects of maternal exposure to *Ricinus communis* oil on morphometric data and anogenital index in F_2 male and female

As shown in Table 4.20 there were no significant differences in the morphometric data at birth in F_2 male and female from gestation days 14-21 treated rats. There were no significant differences also in the anogenital distance and index of male and female F_2 from gestation days 14-21 (Table 4.21).

Table 4.20: Morphometric data at birth in female F_2 pups (from F_1 male and female offspring from treated parents)

Group by gestation day(GD) N=8	WEIGHT**(gm)**	HEAD DIAMETER**(cm)**	ABDOMINAL DIAMETER**(cm)**	BODY LENGHT**(cm)**
CONTROL (FEMALE)	4.65±0.23	1.10±0.02	1.59±0.28	3.95±0.39
GD 14-21 (FEMALE)	4.68±0.14	1.11±0.01	1.21±0.03	4.40±0.03
CONTROL (MALE)	4.74±0.23	1.10±0.02	1.31±0.03	4.31±0.14
GD 14-21 (MALE)	5.08±0.32	1.13±0.04	1.19±0.05	4.58±0.08

Table 4.21: Anogenital distance in male and female (F_2 pups)

Group by gestation day (GD) N=8	AGD**(cm)**	AGD INDEX**(cm/g^3)**
CONTROL (FEMALE)	0.18±0.01	0.11±0.00
GD 14-21 (FEMALE)	0.18±0.01	0.11±0.00
CONTROL (MALE)	0.36±0.02	0.21±0.01
GD 14-21 (MALE)	0.41±0.09	0.30±0.05

4.45 Effects of maternal exposure to *Ricinus communis* oil on morphometric data and anogenital distance in F_2 male and female pups

As shown in Table 4.22 morphometric data were significantly increased ($p<0.05$) in F_2 pups from GD 1-7 and 1-21 when compared with the control. Body weight, abdominal diameter, head diameter and body length were significantly increased ($p<0.05$) for pups from GD 1-7 while weight and abdominal diameter were significantly increased for GD 1-21. GD 7-14 F_2 pups morphometric data were however not significantly different from control. Anogenital distance and indexes of F_2 pups from GD 1-7, 7-14 and 1-21 were not significantly different from control (Table 4.23).

Table 4.22: Morphometric data at birth in male F_2 pups from mating of treated male and untreated female offspring

GROUPS BY GESTATION DAY (GD) N=7	WEIGHT(g)	HEAD DIAMETER(cm)	ABDOMINAL DIAMETER(cm)	BODY LENGTH(cm)
CONTROL	4.74±0.23	1.10±0.02	1.31±0.03	4.31±0.14
GD 1-7	6.90±0.05**	1.20±0.01**	1.53±0.04**	4.90±0.08**
GD 7-14	4.34±0.19	1.06±0.03	1.25±0.02	4.13±0.04
GD 1-21	5.49±0.13*	1.12±0.02	1.43±0.04*	4.42±0.09

KEY: * = p< 0.05, ** =p< 0.01

Table 4.23: Anogenital distance at birth in male F_2 pups from treated male and untreated female offspring

GROUP BY GESTATION DAY(GD) (N=7)	AGD **(cm)**	AGD INDEX**(cm/g^3)**
CONTROL	0.36±0.02	0.21±0.01
GD 1-7	0.39±0.02	0.21±0.00
GD 7-14	0.30±0.02	0.19±0.01
GD 1-21	0.35±0.01	0.20±0.01

4.46 Effects of maternal exposure to *Ricinus communis* oil on body weights at birth and weaning in F_2 pups

There were no significant increases in the weekly average weight among the groups except GD 1-7 F_2 pups that showed significant increase ($p<0.05$) in the average weekly body weight when compared with the control (Figure 4.25)**.**

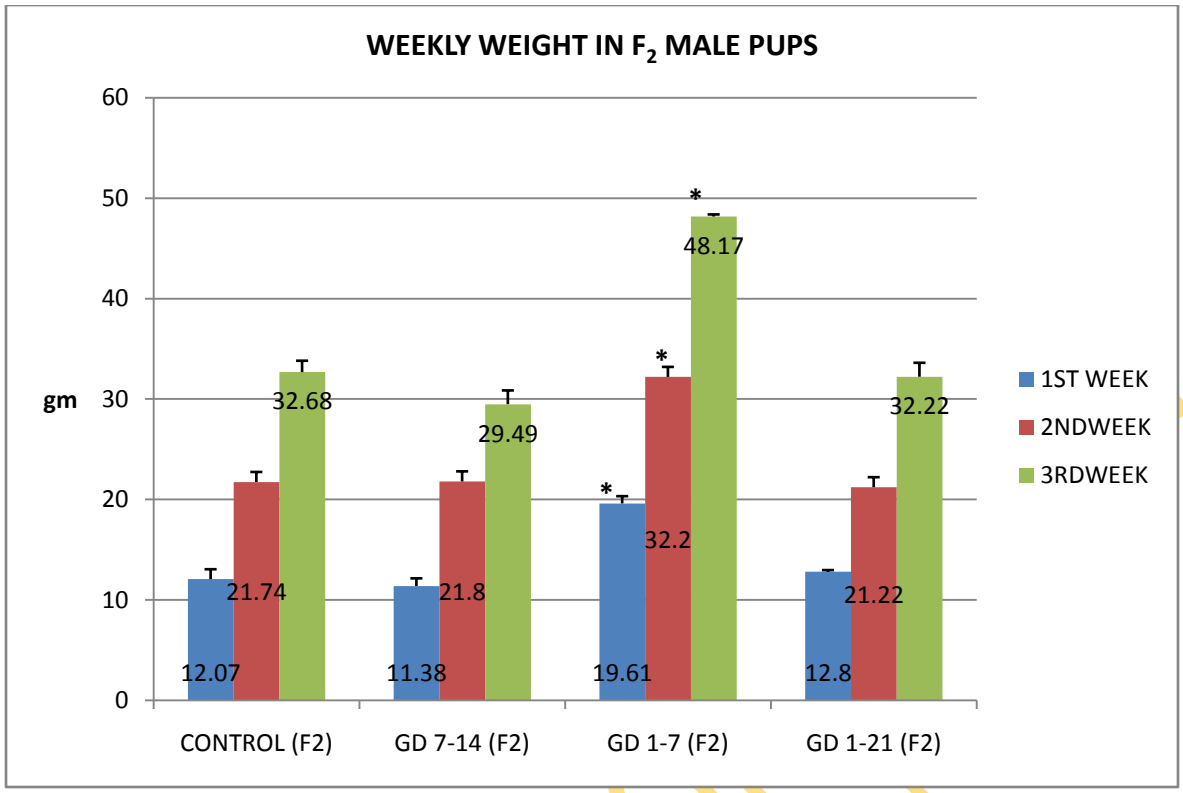

Figure 4.25: Weekly body weight in male F_2 pups from mating of treated male and untreated female offspring

KEY: * = p< 0.05

4.47 Effects of maternal exposure to *Ricinus communis* oil on weight and puberty onset in F_2 offsprings

There was significant increase ($p<0.05$) in the day of puberty onset in F_2 males and females from GD 14-21 (Table 4.24). Average weight and day of puberty onset increased in F_2 males and females from rats treated between gestation days 1-7, 7-14 and 1-21 (Tables 4.25).

Table 4.24: Weight and day of puberty onset in F_2 males and females from (F_1 male and female offspring of treated parents)

Groups(n=8)	Weight(g)	Day
Control(males)	77.0± 1.1	46.8± 1.2
GD 14-21(males)	79.4± 6.2	54.3±1.1**
Control (females)	77.9± 2.2	46.8± 1.1
GD 14-21(females)	64.4± 4.9	53.5± 0.6**

KEY: ** =p< 0.01

Table 4.25: Weight and day of puberty onset in F_2 males and females from (male offspring from treated mothers and untreated female)

Groups(n=8)	Weight(g)	Day
Control(males)	77.0±1.1	46.8± 1.2
GD 1-7(males)	99.4± 2.9**	45.3±0.3
GD 7-14(males)	84.9± 0.6*	51.3±0.3**
GD 1-21(males)	90.2± 3.8**	54.3±0.4**
Control(females)	77.9± 2.2	44.5± 0.5
GD 1-7(females)	101.8± 1.2**	44.8± 0.3
GD 7-14(females)	87.9± 2.6*	53.0± 1.1**
GD 1-21(females)	86.1± 1.8*	51.3± 0.5**

KEY: * = p< 0.05, ** =p< 0.01

CHAPTER FIVE

DISCUSSION

5.1 Route of Administration and Dosage of *Ricinus communis* oil

The route of administration of *Ricinus communis* oil in this study (oral) was in accordance with the route of possible human exposure during pregnancy when used as a laxative or labour inducer. In addition, the metabolic pathway of *Ricinus communis* oil for this route of administration has been extensively delineated and found to be the same in human and rats by previous works (Paul and Mckay 1942, Watson and Gordon, 1962, Thompson, 1980, Ihara-Watanabe *et al*, 1999). Dosage used in this study was also according to recommended therapeutic human dose (Drugstore.com, Inc 2004).

5.2 Study on Physiochemical properties, Gas chromatography and molecular spectroscopy (GCMS) of *Ricinus communis* oil

Values of data (Table 4.1) obtained for acid value, free fatty acid, and density where within the range of those reported from previous studies National Toxicological Programme (NTP), (2003) except saponification value which was much lower than reported values. This could be due to the fact that geographical distribution and individual soil characteristics have been found to influence percentage availability of individual constituents of plant (Rafieiolhossaini *et al*., 2008). The report in this study was the first on the physiochemical characteristics of a typical *Ricinus communis* plant from South West Nigeria. The results from gas chromatography and molecular spectroscopy in this work (Figures 4.1, 4.2, 4.3 and 4.4) corroborates earlier works by Kato and Yamaura, (1970), Larsen *et al*., (2001) and TNO BIBRA International Ltd (1999) alluding to the fact that 80-90% of *Ricinus communis* oil is made up of ricinoleic acid.

5.3 Acute Oral Toxicity Study on *Ricinus communis* oil in rats

There are newer protocols that have been designed in testing for acute oral toxicity of any chemical agents. The newer protocols sought to maximize the total number of animals used and increase the reliability of results from oral toxicity test. These protocols of the Organization for Economic Cooperation and Development (OECD) include the acute toxic class (OECD, 423), the fixed dose procedure (OECD, 420) and the up and down procedure (OECD, 425). For the current study, the agent under investigation i.e. *Ricinus communis* oil (RCO) satisfied the criteria for the use of limit test of the up and down procedure of the Organization for Economic Cooperation and Development (OECD, 425). There were no lethality when animals where dosed at limit dose of 2000mg/kg and 5000mg/kg body weight showing a wide safety margin for *Ricinus communis* oil when ingested orally. Testing at 5000mg/kg body weight was discouraged except for a strong likelihood that such result would have direct relevance for protecting human/animal health and environment (OECD 425, 2001). Availability of RCO as "over the counter drug" (OTC) and the possibility of abuse propelled the test at 5000mg/kg body weight in this study. An acute oral LD_{50} greater than 10g/kg was reported by Allegri *et al*., (1981) for hydrogenated castor oil. In other studies involving incorporation of up to 10% *Ricinus communis* oil in diets, (Masri *et al*., 1962., NTP, 1992, Ihara-Watanabe *et al*, 1999) and intravenous administration of 0.1ml/kg body weight of *Ricinus communis* oil (Lorenz *et al*., 1982), no gross abnormalities or significant effects were observed on survival of groups of male and female rats. This could be attributed to the fact that studies by Watson and Gordon, (1962) indicating greater absorption at lower dose and severe diarrhoea whenever high dose of RCO is ingested.

Thompson, (1980) has also reported that *Ricinus communis* oil was rapidly metabolized by pancreatic lipase leading to release of glycerol and ricinoleic acid.

5.4 Study on Maternal Body Weight, Haematological, Biochemical and Histopathology

This part of the study was undertaken in order to investigate if there were any maternal changes that could subsequently impact negatively neonatal and postnatal growth. The results from these studies showed no significant difference in the body weight of pregnant rats treated with *Ricinus communis* oil at different gestation periods when compared with control. Though there was weight gain, it was not statistically different from that of control. This could be attributed to the fact that feeding habits between *Ricinus communis* oil treated pregnant rats and control were not different throughout the duration of gestation. Both control and treated pregnant rats fed well throughout the duration of the study. This finding is corroborated by studies of Masri *et al*., (1962) and NTP, (1992), where 10% castor oil fed male and non-pregnant female rats for 5 and 13 weeks respectively led to no significant differences in food consumption and mean body weights between test and control groups.

Maternal haematological indices were also not significantly different from that of control in *Ricinus communis* oil treated pregnant rats. Exposure to *Ricinus communis* oil in this study was about one week for each treated group, unlike the National Toxicological Programme, (1992) study where female rats were exposed to 10% *Ricinus communis* oil diets for up to 13 weeks. Despite the long duration of exposure, no haematological abnormality except statistically significant ($P< 0.05$) decrease in reticulocyte counts was reported. The current study is quite

germane as it is reporting for the first time haematological effects in pregnant rats exposed to *Ricinus communis* oil at different gestation periods.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) decreased significantly ($p<0.05$) in pregnant rats exposed to *Ricinus communis* oil at gestation days (7-14, 14-21), and (1-7, &1-21) for ALT & AST respectively. Elevated ALT and AST levels have been implicated in most liver diseases while AST have been found to be mostly of extra hepatic origin with its level rising in heart disease (Pradumna *et al*., 2009). The fact that these two enzymes that are markers of hepato-cellular injury were not elevated showed that RCO has no hepato-cellular or extra hepatic toxic effect. Coupled with this was the fact that liver weight of treated and control rats also showed no significant difference (Table 4.6). Studies by (Masri *et al*., 1962, Lorenz *et al*., 1982, NTP, 1992, Xie *et al*., 1992) have shown that RCO has no acute intravenous, short term oral or sub chronic oral toxicity.

Serum cholesterol levels were significantly elevated ($P<0.05$) in pregnant rats treated with *Ricinus communis* oil when compared to control (Figure 4.7). In a study by Kenji and Nikolaos, (1998) cholesterol enriched diet causing hypercholesterolaemia was found to impair peripheral Leydig cell testosterone responses to testicular stimulation with human chorionic gonadotropin, epididymal sperm content, motility and in-vitro fertilization potential of sperms. Perhaps therefore, the impaired male reproductive parameters and reduced fertility observed later in F_1 offspring in this study could be due to the programming effect of hypercholesterolemia associated with maternal exposure to *Ricinus communis* oil in this study. Optimal Leydig cell function and testosterone secretion are known to be prerequisites for normal activation of spermatogenesis and maintenance of epididymal sperm maturation processes and development of sperm capacity to exhibit vigorous forward progression **(**Jeanne, 1989, Oliviera *et al*., 2001).

Thus the lower epididymal sperm counts and motility profiles in animals with hypercholesterolaemia may be attributable to a defect in the secretory function of the Leydig cells (Kenji & Nikolaos, 1998). Similarly, elevated levels of total lipids observed in this study (Figures 4.8 4.9, 4.10) corroborated the study of Kenji and Nikoloas, (1998) where hypercholesterolaemia was also associated with elevated level of total lipids. Pregnant rats exposed to *Ricinus communis* oil in this study also showed significantly reduced serum levels of progesterone and estrogens (Fig 4.11 and 4.12) when compared to control. Major hormones produced by the feto-placental unit are progesterone, estradiol, estriol, human chorionic gonadotrophin and human placental lactogen (Lording & De Kretser, 1972). The major estrogen produced during human pregnancy is estriol and elevated estriol levels indicate fetal wellbeing. Progesterone and estrogen have numerous beneficial functions in ensuring the survival of the developing fetus throughout gestation period. Estrogen enhances fetal organ development, stimulate maternal hepatic protein production, increase mass of breast and adipose tissue and also increase the size of the uterus and uterine blood flow which are critical in the timing of implantation of the embryo (Rhoades and Bell, 2009). The significantly reduced levels of estrogen in treated pregnant rats in this study with the attendant compromise in the normal function of estrogen might be responsible for the impaired maternal cyto-histology of the uterus, fetal resorptions, deaths (Plates 4.7 and 4.8) and reduced pup weight (Table 4.7 and 4.8) in *Ricinus communis* oil treated offspring particularly during early gestation periods. Progesterone is essential for maintaining the uterus and early embryo, inhibits myometrial contraction and suppresses maternal immunological responses to fetal antigens. Reductions in serum level could be responsible for the impairment observed in the F_1 offsprings in this study. The reduced estrogen and progesterone could however be as a result of *Ricinus communis* oil induced damage

to the uterine wall as shown in plate 4.8 with a subsequent impairment in the normal secretory functions of the uterus that in conjunction with the foetus form the feto-placental unit.

5.5 Generational study on *Ricinus communis* oil

The major goal of the present study was to investigate programming effects on outcomes in male and female delivered from mothers exposed to *Ricinus communis* oil at critical windows of development- early, mid, late and entire gestation periods. From the study of literature, humans (particularly females) are exposed directly or indirectly to *Ricinus communis* oil during these critical periods in the development of a fetus. This study addressed varying questions that remained unanswered from previous studies on effects of maternal exposure to *Ricinus communis* oil by attempting to investigate not only the immediate but long term programming effects on generational reproductive parameters in offsprings maternally exposed to *Ricinus communis* oil.

Histology of the uterus in pregnant rats exposed to RCO at gestation days 1-7 and 7-14 (plates 4.7 and 4.8) showed ballooning of the uterus, uterine tissues disruption, and resorption with implantation sites indicating possible compromise of the uterine support for the developing embryo. These findings could be responsible for reduced litter size and weight experienced in litters from this group in the present study (Fig 4.13, Table 4.7 and 4.8). Fowden *et al*., (2006) posited that changes that could impair intra uterine availability of nutrients, oxygen and hormones usually program tissue development leading to abnormalities later at adulthood. The timing, duration, severity and type of insult during development have also been found to be contributing factor to the type of physiological outcome. From this study, mothers exposed at the early gestation periods have been found to be most susceptible and offspring from them most

compromised. This study also showed that male offspring from gestation days 1-7 and 7-14 are more affected than female. The associations between low birth weight and adult disproportionate phenotype have been linked to poor nutrition and oxygenation during early life because of placental compromise (Harding and Johnson, 1995). Nutrient and oxygen availability have been reported to invariably affect the endocrine environment (Fowden and Forhead 2004). The roles of hormones as programming signals have also been reported in human and experimental animals (Fowden and Forhead 2004). Since *Ricinus communis* oil has been reported to have estrogenic properties (Okwuasaba *et al*., 1991), the impairment observed in the uterus of exposed mothers particularly at gestation days 1-7 and 7-14 and the subsequent reduction in litter size, morphometric data and body weight of pups from this group further validate the assertions of Okwuasaba *et al*., (1991) and Fowden and Forhead, (2004). Low birth weight as a result of disproportionate fetal growth has been reported to be a "classical" risk factor indicating occurrence of fetal programming (Berthold 2007), though other studies have showed programming could occur during pregnancy without influencing birth weight (Nathaniesz and Thornburg, 2003; Berthold, 2007).

The anogenital distance in many species (including rats) provide an excellent non invasive marker of sexual differentiation at birth (Zambrano *et al*., 2006). Androgens (testosterone and dihydrotestosterone) are central and key in that their productions at critical periods of fetal development are essential for proper masculinisation of both internal and external reproductive structures. The central role illustrates why if there is a compromise to androgen as a result of exposure to any estrogenic agents during uterine development it will result in abnormalities of the external genitalia (Mylchreest *et al*., 2002) resulting in reduced anogenital distance. Anogenital distance is a sexually dimorphic secondary sex characteristic in many mammalian

species and can be used to measure the degree of demasculinization of males as a consequence of developmental exposure to androgen receptor (AR) antagonists, 5-alpha reductase inhibitors, or compounds that inhibit steroidogenesis (Fisher, 2004). Likewise, AGD is useful in measuring the degree of masculinization of females exposed during sexual differentiation to androgenic compounds or anabolic growth stimulant. Increased activity and variability in growth rates increase the variability of this measurement. However, decreased male AGD and increased female AGD observed in adult rats at necropsy demonstrate that endocrine disrupting compounds do permanently alter AGD. Anogenital distance is a reliable predictor of permanent alterations of the reproductive system that are not often apparent until the animal reaches sexual maturity. Male pups from mothers exposed to *Ricinus communis* oil in this study showed significant decrease in anogenital distance when compared to control. Clark *et al*., (1990, 1993) reported that male rats exposed in utero to finastride, a 5α reductase inhibitor that blocks conversion of testosterone to dihydrotesosterone displayed decreased anogenital distance at birth, however there was eventual catch up growth of low dose group at adulthood suggesting that decreases in AGD in early postnatal life were transient. Other studies (Gray *et al*., 2000, Mcintyre *et al*., 2002., Ema *et al*., 2003) have also reported reduction in AGD without catch up in adulthood in male rats exposed *in utero* to anti androgens or estrogenic agents. In the present study male rats from exposed mothers at adulthood post natal day (PND) 90 still maintained the reduced AGD observed at birth without any catch up (Table 4.15).

One can surmised that maternal exposure to *Ricinus communis* oil (RCO) resulted in the disruption of the endocrine axis and reduction in the AGD pointing to the fact that RCO has estrogenic effects and could serve as endocrine disruptor *in utero*. To further buttress this fact

there were elevated estrogen levels in F_1 male rats from RCO treated mothers as compared to control at sacrifice on PND 90 in the present study.

5.6 Study on Puberty onset

Vaginal perforations and preputial separation have been considered as good and acceptable marker of puberty onset (EPA, 1996, Ostby and Gray, 2004; Guzman *et al*., 2006, Stump *et al*., 2007). In this study female pups particularly from gestation days 7-14 displayed early and significant onset of puberty (42.20±1.20d) when compared to control (49.00±2.28d). RCO treated male F_1 rats also displayed early onset of puberty when compared to control. Early onset of puberty in this study was related to body weight as both F_1 male and female pups showed reduced weight at puberty. This is in line with other authors who have reported on puberty having relation with body weight (Baker, 1985) while others reported weight as independent of puberty onset (Engelbregt *et al*., 2000, 2002).

There could be early onset of puberty when there is inappropriate gonadal or adrenal steroid secretion (LHRH independent precocious puberty) or hypothalamic luteinizing hormone release (LHRH) pulse generator when pulsatile release of LH that is seen in puberty is inappropriately initiated (central precocious puberty) (Rodney and Bell, 2009). In this type of precocious puberty LH increase in response to LHRH (i.e. LHRH dependent). In this study there was obvious and significant increase in both the serum LH and FSH level (Fig 4.19 and 4.20) in RCO treated F_1 male when compared to control showing either an inappropriate increase in gonadal or adrenal steroid secretion as seen in LHRH independent precocious puberty or inappropriate central pulse generator release of LH as seen in central precocious puberty. The potential of RCO serving as an estrogenic endocrine disruptor sequel to maternal exposure is further exacerbated as findings

have shown that high concentrations of maternal steroids (from endogenous or exogenous sources) can adversely affect both the pituitary-gonadal and pituitary-adrenal functions in the offspring (Guzman *et al*., 2006).

5.7 Fertility in F_1 male and female of *Ricinus communis* oil treated rats

In the present study, F_1 male rats exposed to RCO at different gestation periods showed significant impairment in epididymal sperm characteristics (Figure 4.14, 4.15, 4.26, 4.17 and 4.18). Sperm counts, motility and viability, were significantly reduced when compared to the control. There was also an increase of more than 15% in abnormal sperm cell in F_1 male offspring of treated rats. Similarly serum testosterone levels were significantly reduced in treated F_1 male (Figure 4.22). Histology of the testis and epididymis of F_1 males from RCO treated rats particularly from gestation days 1-7, 7-14 showed interstitial oedema, reduced seminiferous tubular lumen, epididymal hypospermia (Plates 4.12a/b,4.14,4.13c, and 4.19)**.** The reproductive organs weight particularly the testes and epididymis also decreased when compared to control (Tables 4.16 and 4.17). Testosterone is not only key but required in the normal development of the male reproductive organs, so the decrease in weight in this study of the testis and epididymis could be as a result of the significant reduction in the serum level of testosterone. The reduction of testosterone in this study could be as a result of oedema in the interstistial spaces as shown in plate 4.12a/b and 4.13a/b. The accompanying oedema observed in the interstitium could have impaired the normal Leydig cell function and thus reduce the testosterone level. Leydig cell that secrete testosterone are populated in the interstitial spaces (Lording & De Kretser, 1972). Several other studies have also reported deleterious effects of maternal exposure to many agents on the

fertility of male and female offsprings. Stylianopoulou, (1983) reported that maternal adrenocorticotropin injection diminishes reproductive capability in adult life as shown in changes in frequency of copulation and ejaculations. Neonatal administration of testosterone was also reported to affect sexual behaviour and cyclicity in female offspring (Stylianopolou *et al*., 1983). In addition, male offspring of rats stressed prenatally showed decreased sexual activity (Anderson *et al*., 1986) and reduced fertility rate (Zombrano *et al*., 2005b). Dose related decrease in copulation and fertility rates were also reported for male offspring exposed to 5 bromo-2-deoxyuridine, used formally for cancer treatment (Nagao *et al*., 1997). Seiichi *et al*., (2010) also reported that exposing pregnant mice prenatally to carbon nanoparticles impaired male offspring by causing vacoulation of seminiferous tubules and reduction in daily sperm production to about 34% at 10weeks of development. Similarly, male mice exposed prenatally to diethylstilbersterol have been reported to show abnormalities like epididymal cysts, urogenital impairment and enlargements of seminal vesicle (McLachlan *et al* 1975, Foster *et al*., 2011).

5.8 Mating study on *Ricinus communis* oil

As shown in tables 4.18 and 4.19 pairing of F_1 male and female according to treatment group gave a mating index of 100% for all treated groups and control. On the contrary, fertility index was 100% for control and gestation days 14-21 F_1 pair but 0% for other groups. This finding showed that exposure to RCO at early gestation period particularly when reproductive organ are being differentiated will definitely cause the likelihood of infertility later in life. During organogenesis, insults may cause discrete structural defects that permanently reduce the functional capacity of the organ. Rhind *et al*., (2001) have reported that when the insult occurred during gametogenesis, reproductive potential of the next generation may be impaired. However male and female offspring exposed at late gestation periods (i.e GD 14-21) were able to conceive

and deliver safely like control. It may be that exposure after full differentiation of reproductive organ *in utero* did not have any effect in adult fertility potential in later life. Studies (Nathaniesz 1999, Fowden *et al*., 2006, Seiichi *et al*., 2010) have shown that programming effect could be influenced by timing and duration of intra uterine exposure to assault. In addition, there seems to be no adverse effect on libido and copulatory capacity as mating index was 100% indicating non impairment in the central nervous control of sexual behaviour in F_1 male and female. However, impairment in F_1 male sperm characteristics, reduced testosterone level, and impaired histology of the female F_1 adult uterus (plate 4.24a and b, 4.25a and b and 4.26a and b) could synergistically contribute to the 0% in fertility indexes of GD 1-7, 7-14 and 1-21. Interestingly, when F_1 male from treated mothers were mated for a shorter period with untreated female, fertility, gestation and lactation indexes were all reversed to 100% (Table 4.19)

5.9 Second generation (F_2) study on *Ricinus communis* oil

The growing body of both epidemiological and empirical evidences showing that programming effect could be trans-generationally transferred (Nathanielsz and Thornburg, 2003, Drake and Walker, 2004, Ong and Dunger, 2004, Torrens *et al*., 2006) coupled with the effects observed in F_1 from this study was the impetus for this aspect of the study. Nathanielsz, (1999) has identified in his principle of fetal programming that most often than not compensation for disturbances in the fetal environment usually results in secondary and typically negative effects. He also asserted that postnatal effects at compensation or correction may often have further deleterious effects. In line with these two assertions of Nathanielsz (1999), F_2 male and female that hitherto had early onset of puberty in F_1 generation had delayed onset of puberty in F_2 generation (Table 4.24 and 4.25), particularly in female than male. Second generation (F_2) males from gestation days 1-7 also showed significant increase in birth weight as compared to F_1 generation that showed

significant reductions .On the contrary, anogenital distances were not significantly reduced in F_2 rats between treated and control F_2 offspring.

5.10 Conclusions

- Maternal exposure to *Ricinus communis* oil resulted in hypercholesterolaemia and lipid profiles of treated dams.
- Maternal *Ricinus communis* oil exposure resulted in reduced maternal serum progesterone and estrogen
- Maternal *Ricinus communis* oil exposure impaired the histology of the uterus in treated dams
- *Ricinus communis* oil reduced AGD at birth and sacrifice in F1 males- consistent with hypothesized estrogenic effects
- There was imbalance in the reproductive hormonal milieu between treated and control in F_1 offspring at adulthood.
- Sperm parameters, testicular histology and puberty attainment were severely impaired at puberty in RCO treated F_1 male offspring giving credence to the concept of fetal origin of adult diseases
- Data identified the most critical programming period for RCO impairment in reproductive endpoint as GD 1-7 & 7-14.

Exposure to RCO at early gestation periods particularly, programming period for reproductive organ embryogenesis, impaired some estrogenic sensitive reproductive endpoints moreso in male

than in female F_1 rats. This could be attributable to ricinoliec acid and sterol alcohol which from gas chromatography and molecular spectroscopy analysis in this study constitute 80-90% of the fixed oil of *Ricinus communis* seed. Onwuluri and Anekwe, (2001) have attributed that the presence of sterols in RCO is important in that sterols as steroid alcohol are intermediate in the synthesis of related steroids. More so, Green *et al*., (1994) asserted that some steroids have been found to be convertible into animal steroids hormone in the presence of relevant enzymes in-vivo. Thompson, (1980) has also delineated the pathway of enzymatic degradation of RCO by reporting that pancreatic lipase acts on RCO to liberate glycerol and ricinoliec acid. The ricinoliec acid is then rapidly metabolized. Hence, the estrogenic effects of RCO are probably due to sterols and ricinoliec acid present in them. *Ricinus communis* oil actions, consistent with estrogenic agents, seem to be an endocrine disruptor and programmed negatively reproductive endpoints in offsprings.

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