REPRODUCTIVE HORMONE CONCENTRATIONS AND HISTOLOGY OF THE HYPOTHALAMUS, PITUITARY, OVARY, UTERUS AND LIVER OF RABBITS FED ON AFLATOXIN LACED DIETS

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT FOR THE CONFERMENT OF THE DOCTOR OF PHILOSOPHY DEGREE IN ZOOLOGY (ANIMAL PHYSIOLOGY) IN THE SCHOOL OF SCIENCE UNIVERSITY OF ELDORET, KENYA

NOVEMBER 2022

DECLARATION

Declaration by the Candidate

This thesis is my original work and has not been submitted for any academic conferment in any institution; and shall not be reproduced in part or full, or in any form without prior written permission from the author and / or University of Eldoret.

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DEDICATION

I dedicate this work to my immediate loving family that include my dear wife; Mrs Maria Jebiwott Kibet and children; John Kiprotich, Ruth Jerop and Maureen Khamete. Special dedication go to my departed son, the late Isaiah Kiprono, whom God took early in life. He was a major source of inspiration and a lovely jewel to both his parents and siblings. May his soul rest in eternal peace. Dedication is also extended to my loving parents; Leah Tabarno and my late father, Paul Kitilit- the two persons who never spared the rod to create the character that is me and to my brothers, sisters and their entire families, people of substance, faith and courage always.

ABSTRACT

Aflatoxin effect on the liver, results in cellular hepatic damage which inhibit enzyme synthesis or lipid metabolism, causing decreased synthesis of precursor molecules for gonadal and gonadotropic hormones. The presence of aflatoxin in plasma affects animal organ functions and hormone profiles that are related to fertility. The objective of the current study were to determine plasma concentration of oestradiol, follicle stimulating hormone and luteinizing hormone. The other objectives were to determine the histology of the hypothalamus, anterior pituitary gland, ovary, uterus and liver. The association between hormone levels, aflatoxin content and histological changes of organs and quantities of aflatoxin were also determined. The study was carried out using four female New Zealand white breed of rabbits for each of treatments 1, 2, 3 and 4, which had 0, 100, 200 and 400 ppb of aflatoxin in feed dry matter respectively. Blood samples were drawn weekly from rabbits for determination of plasma oestradiol, follicle stimulating hormone and luteinizing hormone using fluorescence immunoassay technique. At the end of the study, one rabbit from each treatment group was sacrificed for the harvest of the hypothalamus, anterior pituitary gland, ovary, uterus and liver. The non-normal distributed continuous hormone level data were log transformed into normal distribution and subjected to analysis of variance and significant means separated using Tukey test. The harvested tissues of the hypothalamus, pituitary gland, ovaries, uterus and liver were processed for histological examination and the results presented in photomicrographs. Mean oestradiol levels were 18.36±7.1, 16.51±6.5, 11.35±3.4 and 7.65±2.9 pmol/ ml, follicle stimulating hormone were 0.52±0.06, 0.38±0.04, 0.32±0.07 and 0.30±0.06 mlu/ml while the mean for luteinizing hormone were 0.38±0.07, 0.26±0.08, 0.190±0.05 and 0.16±0.09 mlu/ ml for each of the treatments 1, 2, 3 and 4 respectively. Aflatoxin caused significant reduction in the plasma concentrations of oestradiol, luteinizing hormone and follicle stimulating hormone in rabbits. The hormone levels were significantly higher in controls compared to animals in treatments groups 2, 3 and 4. The hypothalamus showed degenerative changes, while the anterior pituitary gland did not show aflatoxin induced histological changes at all levels incorporated in the feed, but the ovary, uterus/fallopian tube and liver showed marked inflammatory reactions, cell infiltration and reduced number of follicles in the ovary. The ovarian was covered by dark - red blood - like fluid, an indication of the entry of leukocytes as an inflammatory reaction to the effects of aflatoxin. The liver showed inflamed hepatocyte reaction to aflatoxin. Focal inflammatory areas indicative of lesions were visible in the periportal area of the liver hepatocyte cells. The uterus showed marked defect while the fallopian tube showed vacuolization. In conclusion, aflatoxin caused reduction of oestradiol, follicle stimulating hormone and luteinizing hormone. Aflatoxin in treatment 4 affected the histology of the hypothalamus but had no effect on the pituitary gland. However, it caused inflammation and necrosis on the follicles, uterus and hepatocyte cells. The study demonstrated toxic associations between aflatoxin and hormone levels and organ changes.

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LIST OF ABREVIATIONS

AFB1 - Aflatoxin blue 1(major)

AFB2 - Aflatoxin blue 2

AFG1 - Aflatoxin Green1 (Major)

AFM1 - Aflatoxin in milk (Hydroxylated AFB1)

AMP - Adenosine monophosphate

AOAC- Association of Official Analytical Chemists

Bcl-2 - B-cell lymphoma 2

CDK - Cyclic – dependent kinase

CK1 - Casein Kinase 1

Concentrate- A feed used with another to improve the nutritive balance of the total diet

CYP450- Cytochrome Phosphate 450 multi-species protein enzymes involved in metabolic activities. Those named in this document include CYP1A2, P4501A, P45011B, P45011C, P450111A, P4501VB, P45011A, CYP3A4

DNA - Deoxyribonucleic acid

ER_{α} - Estrogen Receptor alpha

ER_{β} - Estrogen Receptor beta

FAO - Food and Agricultural Organization

FSH - Follicle Stimulating Hormone

FDA - Feed and drug administration

GABA - Gamma – Aminobutyric acid

GnRH - Gonadotropin releasing hormone

IARC - International Agency for Research on Cancer

IGF - Insulin growth factor

IITA - International Institute of Tropical Agriculture

KALRO- Kenya Agricultural and Livestock Research Organization

KBS - Kenya Bureau of Standards

LH - Luteinizing hormone

MLU/ml- mili-international units/ mililiter

NF-Kβ - Protein complexes that control transcription of DNA, cytokine production and cell survival

NMDA- N – Methyl – D - Aspartate

PACA - Partnership of Aflatoxin Control in Africa

PPb - Parts per billion

PPM - Parts Per Million

RNA - Ribonucleic acid

RPM - Revolutions per minute

USA - United states of America

UV - Ultra Violet

WHO - World Health Organization

ACKNOWLEDGEMENT

All gratitude and adoration go to the Almighty God who gave me good health, resources, strength and persons who assisted during the study. I also wish to sincerely thank individuals and organizations who contributed to the success of this work. Sincere thanks go to my University supervisors; Dr Dickson M. Mwaniki and Prof. Gideon N. Magak for their in-depth and incisive guidance during the study and reporting of the findings. Much appreciation go to individual members of staff of Moi Teaching and referral Hospital, specifically Dr Moses Kiplel Ndiema, David Chumba and David Chesire for their detailed analysis of various samples from the rabbits. I also thank Nairobi Hospital Annex at Eldoret for helping in hormone analysis and Leonard for his meticulous techniques in the bleeding of the rabbit.

I highly thank Bora Biotech Ltd for preparing the aflatoxin laced feeds for the study. Robert Kiptoo braved the rigour of attending to the experimental animals throughout the study period, particularly for keeping the place clean and the rabbits fed as stipulated, and for this, I am grateful. Special recognition is also go to members of my family for their encouragement, patience and prayers. I recognize their sacrifices which enabled me to get the funds for the study.

CHAPTER ONE

INTRODUCTION

1.1 Background

Mycotoxins have been known to contaminate about 25% of the world's food supply each year, particularly cereals and groundnuts that are produced for consumption and approximately 1 person in 100,000 people are exposed to aflatoxin poisoning in the world each year (WHO 2018; Wu *et al*., 2013). The factors influencing aflatoxin production include favourable temperature range between 24 to 35^{o}C and relative humidity of 58 to 70% which, in most cases is rampant within tropical regions (WHO 2018).

Aflatoxins are poisonous products of moulds or non-mould fungi found in grains, grass hay or legumes harvested and stored under moist conditions. Some feeds may be more prone to contamination, particularly cereal grains, grain by–products, protein concentrates, compounded rations, oilseed cakes and forages. The presence of aflatoxin in food and feeds is often overlooked due to lack of public awareness of their existence, lack of sufficient regulatory mechanisms, dumping of food products by importers and the introduction of contaminated commodities into the human food chain during chronic food shortage arising due to drought, war, political and economic instability (Muthomi *et al*., 2009).

High concentrations of aflatoxins above acceptable levels are in some instances found in feed grains that include maize, groundnuts and other cereals in the tropics (Kitya *et al*., 2009). They are secondary metabolites that pose serious hazards to animal and human

beings (Rustemeyer *et al*., 2010; Jamal *et al*., 2012). The severity and effect of poisoning depends on age; younger animals are more prone than mature ones: while different sexes of particular species of animals are affected differently, the amount of aflatoxin being consumed and duration of exposure influence their effects too (Gugnani, 2000).

Aflatoxin in sera, food or feed causes harmful changes in animal tissues as reported by WHO (2018), but was not specific whether it meant reproductive tissues and organs. High enough exposure levels are fatal for mammals, birds, fish as well as human beings. The liver is the principal target organ affected, but high levels have been found in the kidneys, lungs and even the heart of individuals that died of acute aflatoxicosis. In the liver, AFB1 is biotransformed by microsomal cytochrome P450 enzymes to a highly reactive intermediate, AFB1-8, 9-epoxide which binds to nucleic acids to form adducts (Sun *et al*., 2015).

Acute necrosis and cirrhosis of the liver, with some level of haemorrhage and oedema have been reported (Eze *et al*., 2018). Chronic effects occur following long term consumption of small amounts over time and the liver again is the main target which, may result into liver cancer, chronic hepatitis, jaundice, cirrhosis and impaired nutrient utilization (Eze *et al*., 2018). Aflatoxins are invisible, highly corrosive chemical poisons to the hepatic and reproductive systems and are carcinogenic to various body organs (Kumar *et al*., 2016). It has been reported that their presence disrupts various cellular structures of the liver, intestinal mucosa and interferes with vital reproductive physiological processes in the

animal's body (Sharma 2011). Aflatoxin binds and interferes with enzymes and substrates that are necessary in the initiation, transcription and translation processes that play key roles in protein synthesis in the rabbit's body. It also interacts with purine and purine nucleosides and temper with protein biosynthesis through formation of adducts with DNA, RNA and proteins (Sharma 2011). Aflatoxin inhibits RNA biosynthesis by interacting with DNA-dependent RNA polymerase to cause degranulation of endoplasmic reticulum. It is thought that the reduction in protein content in body tissues as is the case in skeletal muscle, heart, liver and kidney could be due to increased liver and kidney necrosis (Sharma 2011).

According to Adedara *et al*. (2014), aflatoxin does not only end up disrupting animal cells and reproductive system, but get concentrated in meat, eggs and milk where it poses a threat to human health. Significant reproductive health have been implied for animals consuming diets contaminated with aflatoxin without specific organ effect except that on the liver (Adedara *et al*., 2018). Much work has been carried out to investigate the effect of aflatoxin on reproductive health of various animal species, but the studies have mainly centered around male spermatozoa production and viability, the cells of leydig and secretion of testosterone, but in female, it centered on oestrogen and progesterone production (Ibeh & Saxena 1997, Adedara *et al*., 2014, Koutousekos *et al*., 2015, Sheikhpour & Jabari 2015, Amin *et al*., 2019).

Aflatoxin in plasma has been reported to cause epigenetic effect on hormone that is related to reduction in animal fertility, cause abnormal function of reproductive organs, lowered overall fertility of animals, particularly causing low quantities of testosterone in sheep (Sheikhpour & Jabari 2015) through direct action on hormones and hormone receptors.

Aflatoxin has been reported to pose carcinogenic risks (IARC, 2012), hepatotoxic and serious hazard to both animals and human beings (Sidhu *et al*., 2009; Rustemeyer *et al*., 2010). Frequently isolated species of fungi in both human and animal feed stuff are mainly *Aspergillus flavus* and *Aspergillus parasiticus,* which are the main fungus producing aflatoxin (Hasanzadeh *et al*., 2011). According to this author, little has been documented concerning the effect of consumption of moderate levels of aflatoxin, which is a common occurrence in subtropical and tropical regions. Its presence in feed, particularly the contaminated concentrates and hay is in most cases higher than the permitted levels of 50 ppb (WHO 2018), making it necessary to establish the possible effects on the various female reproductive organs.

Consumption of aflatoxins in diets exposes individual animals to health problems which can result into unexpected health outcomes. Aflatoxicity can be either acute or chronic, and its outcome on both human and animal health may vary; but mainly cause major damage to the liver and other body organs, and has also been reported to damage reproduction in animals (Kowalska 2017). The exposure of aflatoxin to animals has been linked to its interference with the production of reproductive hormones and interaction with steroid

receptors (El-Shahat *et al*., 2012). Aflatoxin may affect the reproductive system by its toxic effect on the liver, where cellular hepatic damage could inhibit enzyme synthesis or lipid metabolism, causing decreased synthesis of precursor molecules for steroid, gonadal and gonadotropic hormones function (Handan & Güleray, 2005).

The interference with the production of reproductive hormones as stated by El-Shabat *et al*. (2012) has not been quantified, and the current study wished to show what the different aflatoxin levels can do to the concentration of reproductive hormones and the organs related to reproduction. Animals fed on conserved feeds have currently shown long post-partum interval, delayed cyclicity and there is a possibility of showing reduced conception rates. Mahady *et al*. (2015) hypothesized that the delay in oestrus cycle in animals might be due to aflatoxin inhibition of hormone function and delay of cellular production by organs of reproduction.

These effects form the area of knowledge concern for the current study because it is hypothesized that aflatoxin's interferes with protein synthesis, hormone secretion and damage of important organs and tissues of reproduction in an animal's body.

1.2 Statement of the Problem

The normal rabbit litter size has been shown in the recent past to be from 7 – 10 Kittens per litter and the ability of the rabbit to return to reproduction as soon as the male is paired/introduced to the female. These rabbits have high conception rates to facilitate the kiddling of up to 10 Kittens and a total of over 700 Kg of meat in a year. But the current state indicates a situation where litter size is as low as 3 – 6 kittens/ litter and the rabbit ovulation frequency and conception rates have declined over time, leading to increased generation interval (Mailafia *et al*., 2010). Aflatoxin could be one of the causes of this low litter size reduction and decline in conception by interfering with hormone secretion or damage on organs that play important roles in reproduction. Aflatoxin has been reported to diminish the fertility of oocytes by disrupting with their maturation by way of epigenetic modification and cause oxidative stress, high autophagy and a its presence in feed, particularly the contaminated concentrates and hay is in most cases higher than the permitted levels of 50 ppb (WHO 2018).

Animals fed on conserved concentrates and forages have in the recent past shown long post-partum interval, delayed cyclicity and there is a possibility of showing reduced conception rates. Mahady *et al*. (2015) hypothesized that the delay in oestrus cycle in animals might be due to aflatoxin inhibition of hormone concentration and delay of their cellular production by organs of reproduction. The Food and Agriculture Organization (FAO) estimated that 25% of the world's food crops are affected by mycotoxins, of which

the most prevalent is aflatoxin, which is known for its negative effects on animal reproduction (FAO 1997).

1.3 Justification

The current study sought to establish whether up to 400 ppb of aflatoxin consumption by rabbits, have effects on reproductive hormone secretion, damage on organs that determine reproduction and the overall effect on reproductive efficiency among domestic rabbits. This study arose due to earlier work on investigation of commercial concentrates in urban Centres in Kenya revealed that most feeds contain up to 550 ppb of aflatoxin (Kangethe & Lang'a, 2009). This were too high compared to the allowable quantities for rabbits which is 50 ppb. Feed samples taken by this author, showed that 81% of the feed were positive for aflatoxin, with Nakuru and Nairobi urban centres showing an average of 280 ppb of aflatoxin B1 alone. The current study was aimed at establishing the effects of aflatoxin around these levels on both reproductive hormone levels and possible damage on organs that play important role in reproduction, which would determine the kitten litter size. The information generated from the study will be useful to Scientists, animal feed manufacturers and feed regulatory institutions with information which will enable them to produce feeds with safety considerations for effective reproductive health.

Decline in reproduction among domestic animals reared intensively has been observed by animal owners and the current study seeks to establish whether there exists links between such declines in performance with aflatoxin. It is necessary to establish the effects of

aflatoxin on organs that play important roles in secretion of reproductive hormones so that corrective and preventive measures can be put in place by feed manufacturers, regulatory bodies and rabbit farmers.

Cholesterol is a major precursor of the steroid hormones, which regulate the main physiological functions in an animal's body, including the reproductive system. This is because, low levels of cholesterol in an animals' body may result into low synthesis of steroid hormones, whose result is poor reproductive performance.

Animals that consume aflatoxin contaminated feed pass the metabolite residues through animal products such as milk, meat and eggs to human beings, passing on aflatoxin M1 which has been reported to cause suppression of protein synthesis and growth among children ((Neyole *et al*., 2008; Applebaum *et al*., 2003).

Several small scale feed manufacturers have been started to compound commercial concentrates for animal producers in the recent past. Most of these manufacturers are oblivious of the need to produce feeds with minimum aflatoxin content or they choose to avoid the cost involved the production of feeds that are free of aflatoxin. This being the case, their feeds contain varied amount of aflatoxin which may affect animal reproduction.

New Zealand breed of rabbits were preferred for the current study because it has served well as an animal model, its medium size and the expected fast physiological changes and its genetic pre-desposition simulates those of other animals. The litter sizes born and

kindling interval among rabbits in Kenya are low. This warrants the study on possible aflatoxic effects on hormone levels and damage to organs that play major roles in female reproduction.

1.4 Limitations of the study

The first of these was due to the single breed of rabbits, New Zealand white, used for the study. Aflatoxic effects have been reported to be influenced by animal breed, species and age of animals among other factors. The effects reported in the current study may not be applicable to all animals in equal measure because of the influence that animals genetic characteristics and body condition have on aflatoxic metabolism(FAO, 2000).

Female rabbits are known to be male rabbit induced ovulators, which ovulate 30 minutes after mating, and without which, ovulation cannot occur. The current study did not include a buck in any of the rabbit pens. This could not jeopardize the findings, however, because the other parameters of the study that included rabbit growth, follicular development and ova atresia were observed without requiring any ovulation. The other limitation was none determination of the effects of aflatoxin on specific enzyme enzymes both at the gastrointestinal tract and the liver.

1. 5 Objectives

1.5.1 General objective

To evaluate the plasma concentration of oestradiol, FSH and LH and histology of the hypothalamus, pituitary gland and ovary uterus and liver in New Zealand white rabbits fed on diets laced with different levels of aflatoxin

1.5.2 Specific objectives

1. To determine the plasma levels of oestradiol, follicle stimulating hormone and luteinizing hormone in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin
2. To assess the histology of the hypothalamus, anterior pituitary gland and ovary in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin
3. To determine the follicular morphology in female rabbits fed on diets laced with varying levels of aflatoxin
4. To investigate the histology of the uterus, fallopian tube and liver in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin
5. To determine the association between the hypothalamo – pituitary - follicular cell changes and the oestradiol, Follicle stimulating and luteinizing hormone

levels. Secondly, the aflatoxin level vis-a-vis the levels of oestradiol, Follicle stimulating hormone and luteinizing hormone levels

1.5.3 **Hypotheses (Ho)**

Ho1. There is no variation in plasma levels of oestradiol, follicle stimulating hormone and luteinizing hormone in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin

Ho2.There is no histological difference of the hypothalamus, anterior pituitary gland and ovary in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin

Ho3. The follicular morphology in female New Zealand White rabbits fed on diets laced with different levels of aflatoxin did not differ.

Ho4. The varying concentrations of aflatoxin in rabbit diets had no effects on the histology of the uterus, fallopian tube and liver in female New Zealand white rabbits.

Ho5.There was no association between the hypothalamo-pituitary-gonadal axis tissue changes and the plasma oestradiol, Follicle stimulating hormone and luteinizing hormone levels and secondly aflatoxin level vis-a-vis the levels of oestradiol, Follicle stimulating and luteinizing hormone concentration

1.6 Conceptual Framework

A conceptual framework involving both independent and dependent variables tested in the research process, which may require more than one notion to test, and the ideas need to be identified as concepts that are understood (Ivey 2015).

Intake of aflatoxin intoxicated feed by animals and the subsequent metabolic series of reactions both at the digestive system and the body cells are directed by physiological processes as dictated by homeorhetic controls under normal circumstances. The presence of aflatoxin in the diet consumed can alter the initiation of the physiological processes through the limitation of the supply of sufficient nutrients or through injuries of organs that play vital roles, including the production of hormones and enzymes.

Aflatoxin has the potential to cause stress in animals through interference on its histology and possibly endocrine system and the hypothalamo- pituitary- gonadal axis and at the process may militate against its reproductive performance.

In case the effects of aflatoxin are minimum or nonexistent, then the animal physiological process will proceed uninterrupted, and for the current situation, reproduction will occur.

Figure 1.1 is a theoretical concept with an attempt to show the possible interference of aflatoxin that may result into impaired reproduction

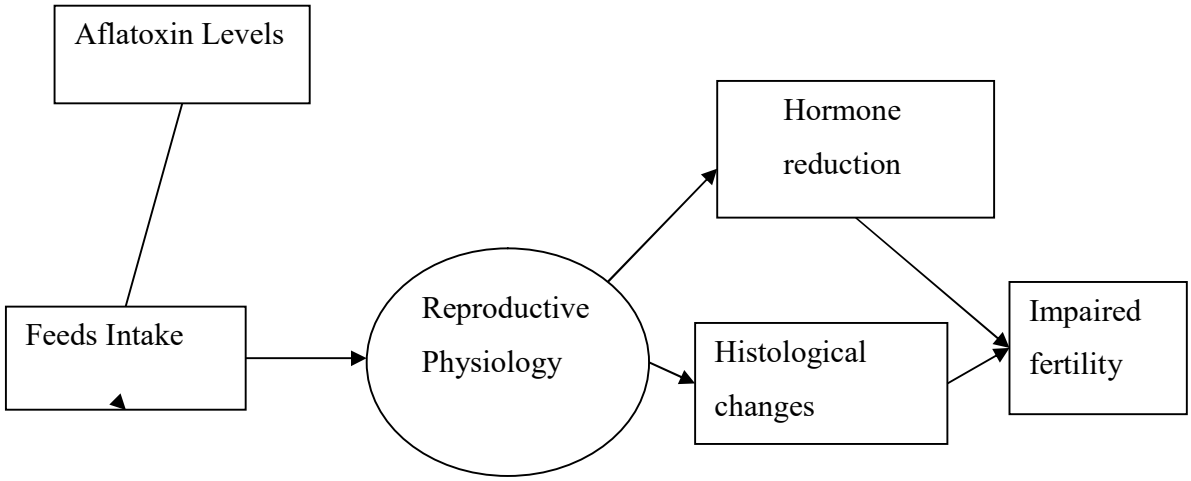


Figure 1.1: Conceptual framework of the effects of aflatoxin on hormone levels in rabbits

The arrows in figure 1.1 represent the effect of one variable on the other, while lines are indicative of correlations of variables.

Aflatoxin has the potential to cause hormone reduction, physiological disorders and histological changes in animals whose overall result is reduced production of milk or meat (Boonyaratpatu *et al*., 2002). Aflatoxins exert their effects on livestock through alterations in nutrient content by way of reactive attachments- especially to unsaturated nutrients, nutrient absorption when the mucosa of digestive tract is corroded and metabolic changes through alteration of endocrine and neuroendocrine functions (Waldner & Lalman, 2010).

The other important factors affecting fertility are development programs, disease incidences like cystic ovaries and endometritis, and the prevailing environmental variables, particularly management (Aiken *et al*., 2015).

Decline in reproduction among rabbits reared intensively has been observed by stock owners and the current study seeks to establish whether there exists links between such declines in performance with aflatoxin. It is necessary to establish the effects of aflatoxin on organs that play important roles in secretion of reproductive hormones so that corrective and preventive measures can be put in place by producers and other stakeholders.

CHAPTER TWO

LITERATURE REVIEW

2.1 Rabbit Reproduction

The mammalian reproductive process begins at the olfactory system through detection of non-volatile pheromone cues from conspecifics that influence several aspects of their social communication, specifically sexual attraction, courtship and mating, and the subsequent release of gonadotropin hormones from the pituitary gland. Evidence has shown that the main olfactory system detects a variety of volatile odorants that work as pheromones to enable mate recognition and activation of the hypothalamic-pituitary-gonadal neuroendocrine axis for hormone discharge and exhibition of reproductive behaviour (Baum & Cherry 2015).

Reproduction in animals is influenced by nutrition among other factors and may involve the influence of growth, fertility, mating behavioral activities and pregnancy. An imbalanced intake of energy and proteins results in severe obesity or underweight which affect ovarian function and may cause increased infertility that is normally associated with low energy content and limited intake of nutrients, particularly microelements (Silvestris *et al*., 2019). A change in energy intake that results in over or under weight has been reported to be responsible for the disorders observed during ovulation (Priveral *et al*., 2018). According to Silvestris *et al*., (2019) overweight and obesity significantly minimize pregnancy rates and increases abortion though there is increased gonadotropic hormones.

Higher consumption than optimum amounts of carbohydrates, proteins/vitamins and micronutrients results into negative impact on ovulation, but normal nutrient intake have a positive impact on oocyte development and maturation of embryo with optimum implantation efficiency of the conceptus (Sivestris *et al.*, 2019). The nutrients are required in optimum amounts to improve fertility particularly the supply of proteins, vitamins, microelements and sufficient amount of energy (Jokela *et al*., 2018).

2.1.1 Regulation of Reproduction

Reproduction as a means of perpetuation of animal species which is important for the existence of life and species immortality. Different animal species have different methods of reproduction, which are also regulated in several different ways. Reproduction is important among mammalian domestic animals species as a means of producing products for its offspring and incidentally for human consumption. Fertility is an important parameter of productivity because in rabbit animal species, the number of off springs born per parturition and their regularity are an indicator of high productivity (Petrovic, 2000). Rabbit fertility and the factors that influence it, along with the liter size are matters of concern to producers. Reproductive life starting from the onset of puberty, fertility rating and twinning rate are important factors that contribute to the productivity of an animal (Robson & Smith 2011). The other important factors affecting fertility are development programs, diseases incidences like cystic ovaries and endometritis, and the prevailing environmental variables, particularly management (Aiken *et al*., 2015).

Genetics play a very important role in reproduction of all animals and any genetic malfunctions have been estimated to cause about 50% of infertility cases. Numerous experiments using animal knockout models strongly pointed to infertility as arising from single or multiple gene defects (Zorilla & Yatsenko 2013). However, the specific genetic causes have not been explained for the large population of male and female infertility cases in rabbits. The reason being the large genes to be examined; estimated at 2,300 genes for human beings for example, which are expressed in male testis, and hundreds of these genes participate in reproductive actions and may each contribute to male infertility (Zorilla & Yatsenko 2013). Currently, a few genes or genetic abnormalities have been demonstrated to play a major role, or are highly correlated to primary infertility among specific animal species. It is however believed that upon the advanced identification of human genes and the development of personalized conventional medicine, the status may quickly be improved and be able to identify the various genes affecting fertility of various other animals (Zorilla & Yatsenko 2013). Genetic components have been reported to affect the fertility of animals, where in extreme cases, some may be infertile while others may be highly fertile as for example; the Romanov sheep breed, which has been reported to be highly fertile such that their lambing percentage goes up to 250%, while Pramenka and the majority of the other sheep breeds have lambing of 110%, but genetic mutation can alter this proportions (Petrovic *et al*., 2012). Among the majority of the other sheep breeds, nutrition, which is an environmental factor has a lot of influence on fertility rating.

Genetic effects on a number of traits related to reproductive life and disease incidences have been reported to affect animal fertility (Laisk-Podar *et al*., 2015). Genetic contribution to reproduction and disease incidences arise from gene mutations at certain sites and variations, which may be observed at different sites of the genome indicating minor defects. In the last ten years, genome-wide association studies (GWAS) have elucidated the Knowledge on genetic functions to explain the complicated trait and disease incidences (Day *et al*., 2015). The results of these studies have led to the discovery of novel genes and pathways influencing specific traits and diseases, new discoveries in disease epidemiology, and the discovery or repurposing of candidate therapeutics (Day *et al*., 2015).

Rabbit practices such as handling, housing or transportation, which are essential in the wellbeing of animals, can be stressors that cause disturbances and increase the release of the hormone cortisol, which has been shown to increase anxiety and decreased reproductive performance (Bristow *et al*., 2006; Cooke *et al*., 2012). Stresses caused by housing include inadequate ventilation, cooling and the regrouping and high density, which cause increased cardiovascular function, neurologic dysfunction and result into reduced rabbit fertility (Sapolsky, 2005).

Nutrition controls hormonal systems with an impact on reproduction, regulation of seasonal reproductive behaviour and is the main cue controlling fertility in cyclic animals. It is the major determinant of reproductive efficiency factor in different animal species. According

to Lassoued *et al*. (2004) important interactions between genotype and level of nutrition, where higher level of nutrition prior to and during mating was associated with improved reproduction. An optimum nutritional status and energy reserve are necessary for hypothalamic – pituitary gland – gonadal integrity and has great influence on endocrine systems, where the beneficial effects are seen through multi-hormonal actions that include gonadotropic and metabolic hormones (Bova *et al*., 2014; Abadjieva *et al*., 2011). A functional defect on any one of the components of this hormonal complex, affects reproduction directly (Petrovic *et al*., 2012). Body reserves and feed intake contribute to the nutritional feedback to the hypothalamus, although reproductive neuroendocrine output from GnRH/LH, is more stimulated by feed intake than by adipose tissue (Petrovic *et al*., 2012; Forcada *et al*., 2006). Findings by Meza-Herrera & Tena–Sempere (2006) showed that nutrient supplementation for 4 – 6 days at pre-ovulation increases an animal's ovulation rate by 20 – 30% without detectable change in body weight or body condition.

The control of the hypothalamo-pituitary-gonadal axis is facilitated by hormone, neuron and glial cell regulations that facilitate reproductive activities (Christensen *et al*., 2012). The neuro-endocrine regulation results into a single product release of gonadotropic releasing hormone, which is also influenced by coordination of internal and external factors. The effect of each environmental signal varies among organisms where some are highly responsive to day length, fluctuation in temperature, olfactory input, nutrient availability, light or acoustic stimuli and internal signals such as steroid hormone feedback and hormone controls (Christensen *et al*., 2012). Both external and internal cues converge

to regulate the GnRH generator for transduction of the cues into reproductive output according to Christensen *et al*. (2012).

In female rabbits, internal hormonal changes can be interfered with by external factors that bring about reproductive changes. The hormone oestradiol plays a central role in the control of internal reproductive environment of female rabbits. The ovarian synthesis of oestradiol that is produced by secondary follicles is regulated by feedback mechanisms coordinated between the hypothalamus, anterior pituitary gland and the ovaries. This oestradiol signal regulates the neural network by either facilitating or inhibiting the release of GnRH to the pituitary gland, causing it to synthesize and discharge follicle stimulating hormone, luteinizing hormone and causing ovulation in the animal (Christensen *et al*., 2012). The release of GnRH is triggered by a positive feedback loop involving oestradiol concentration which activates the neuropeptide kisspeptin that is vital in the discharge of GnRH (Millar *et al*., 2010). Gamma-aminobutyric-acid (GABA) containing neurons also enhance the release of LH via the control of catechol-aminergic system that controls GnRH secretion and neuro-steroid actions through NMDA receptors (Giuliani *et al*., 2011).

Dysregulation of ovulation occurs also under disease situations particularly conditions that affect the ovaries. The development of cystic ovarian disease in cattle has been found to cause increased LH, which is associated with the attempt by the hypothalamic-pituitary-gonadal axis to maintain homeostatic control of the circulating hormones in plasma (Christensen *et al*., 2012). The increased LH release is related to alterations in the

expression of estrogen receptor alpha (ER_{α}), estrogen receptor beta (ER_{β}), androgen and protein receptor, where high expression of ERα in the granulosa cells and the cells of cystic ovary is associated with increased LH secretion (Alfaro *et al*., 2011). According to Vanholder *et al*. (2006), animals with cystic ovaries present high levels of circulating oestrogen, LH and altered expression of steroid receptors at the ovary. This implies a situation of anovulation due to alterations in steroid receptor due to the effects of the disease. In most females, the production of large quantities of GnRH in form of a surge, results into corresponding peak in LH, which stimulates the production of Oestradiol from theca internal and luteal cells (Williams 2013). Reports by Williams (2013) showed the down regulation of LH by placental oestradiol due to reduction of neuroendocrine signals that minimize gonadal functions in lactating rabbits. The same author attributed this to depletion of LH stores in the pituitary gland by parturition resulting in the inability of hypothalamic centre to respond to positive feedback effects of oestradiol within 4 weeks after kidding in suckling rabbits.

Suckling has been reported by McNelly (2012) to increase the sensitivity of hypothalamus to negative feedback effects of oestradiol by suppressing the GnRH/LH pulse generator among several animal species. Suckling stimulus provides the controlling signal in breast feeding but a return to normal cycles follows a pattern from complete inhibition of GnRH/LH pulsatile secretion during early stages of lactation to erratic pulsatile secretion

with ovarian follicle development associated with increases in inhibin B and oestradiol to normal follicular growth due to increase in oestradiol (McNelly 2012).

2.1.2 Role of Hypothalamus and anterior pituitary gland in reproduction

The hypothalamus plays an important physiological role in the body through its influence on both the endocrine and nervous system of an animal. It maintains fundamental internal body physiological programs that include body temperature, eating, sleep, sexual behavior and emotional behavior; including pleasure and rage through stimulation or inhibition of key body processes. The various sub-regions of the hypothalamus play distinct roles in its reaction to both internal and external stimuli, with others increasing in action as others diminish activities including reduction of cardiovascular stimulation (Fontes *et al*., 2011).

In the reproductive front, the preoptic part of the hypothalamus, through the stimulation of both internal and external cues, produces GnRH, a peptide neuro-hormone, secreted in specific neural cells and released at the neural terminal into the anterior pituitary gland (Kunimura *et al.,* 2015). The neuro-hormones, produced from the median eminence of the hypothalamus, are transported to the capillaries of the portal veins of the anterior pituitary gland to regulate by inhibition or stimulation the secretion of specific tropic hormones (Kunimura *et al.,* 2015).

Secondly, the hypothalamus secretes the neuro-pituitary hormones that are transported via the long axons of the hypothalamo-pituitary tract where they are stored in neural lobes of the hypophysis and released later into circulation when need arises (Kunimura *et al.,* 2015).

The pituitary gland also referred to as adenohypophysis, is a tiny gland weighing about 0.5 to 1.0 gm and is located at the *sella turcica* at the brain cavity, where it is connected to the hypothalamus by the hypophyseal stalk (Guyto & hall, 2011).

The anterior pituitary gland secretes thyrotropin and growth hormones whose purposes, among others, include the increase of blood glucose levels in the animals through various metabolic pathways, and secretes Corticotropin hormone also, which causes the adrenal cortex to initiate the secretion of Adrenal corticoid hormone (Choi & Smitz, 2014).

In reproduction, the anterior pituitary gland secretes prolactin hormone whose function is to cause the development of the mammary gland and multiplication of alveoli cells in preparation for milk secretion and lactation of the animal. The pituitary gland also secretes gonadotropic hormones; the follicle stimulating hormone and luteinizing hormone, which are important for the development, maturation and rapture of the follicles in readiness for ovulation (Choi & Smitz 2014). Both follicle stimulating hormone and luteinizing hormone are released from the pituitary gland to cause maturation of primordial follicles through the stimulation of GnRH, but inhibited by oestrogen surge in a negative feedback mechanism (Esparza *et al*., 2020). The presence of high LH following the maturation of primordial follicles, triggers the synthesis of steroid hormones in the ovaries (Choi & Smitz 2014).

2.1.3 Role of ovary and uterus in Reproduction

The ovaries are paired organs that serve both gametogenic and endocrine production functions in most animals. These dual roles are complementary, interdependent and

necessary for success in animal reproduction. The ovarian follicles, contained in the ovaries, are basic functional units in mammals that display unique development trends and manifest independent functions in the ovary (McGee & Hsueh, 2000). The follicles themselves are sacs filled with fluid at the atrium and whose two main functions are oocyte maturation and release, and steroid hormone synthesis which play an important role in growth and creation of suitable uterine environment for the gametes (Ernest *et al*., 2017). Mammalian adult ovary, has a reserve of primordial follicles containing dormant oocyte and pregranulosa cells containing follicular lamina. Primordial follicles are regularly activated to function throughout the life span of an animal through pathways involving growth factors like P13K/PTEN/Akt (Ernest *et al*., 2017). The action of P13K/PTEN/Akt pathway determines the sizes of primordial follicles. Dominant follicles grow faster on a daily basis, whether it has a viable oocyte or not, they have more fluid in the atrium, which contains high amount of oestrogen and has a high synthetic capacity for latter hormone (McGee & Hsueh, 2000; Ryan *et al*., 2007). A number of primordial follicles undergo atrecia any time they are not expanding (Rodger & Irving-Rodger, 2009).

The Uterus is important as it serves as a tubular structure for the transportation of spermatozoa into the oviduct where fertilization takes place and latter receives the embryo for implantation to the uterine wall to begin the gestation period. It is a vital structure of reproduction where the conceptus is implanted, nourished and develops into a foetus during gestation. Any damage to the structure has the potential to impair normal uterine function,

and may result into abortion or embryonic death culminating into failed reproduction of the animal or low birth weights of the offspring (Shaw *et al*., 2010). The implantation process, likewise, requires primarily, the support of sufficient quantities of oestrogen and progesterone hormones, although other hormones may be at play in the control of a host of cells to carry out the implantation, growth and development process of the foetus (Shaw *et al*., 2010).

2.1.4 Seasonal influence on reproduction

The rabbit's natural selection encourages its adaptation to the environment and at the process favours reproduction to take place in harmony to the environmental cues that are normally in concurrent with seasonal alterations of some kind, just as manifested in animal trends characterized by alignment towards reproductive functions (Bronson & Heideman 1994). Seasonal influences on reproduction can be observed through changes in the number of animals indicating sexual activities within the season. Though, it can include an associated number or the percentage of mature animals ovulating and the rate of ovulation.

The changes in seasons influences reproduction of vertebrate animals through effects of the hypothalamic–pituitary–gonadal axis. The release of gonadotropin-releasing hormone from the hypothalamus causes the subsequent secretion of luteinizing hormone and follicle-stimulating hormone (FSH) from the hypophysis, which targets the gonads for reactivation and maturation of follicles. The hypothalamic–pituitary–gonadal axis of seasonal breeding females is reactivated at the right season for breeding as dictated by the climatic cues. In

the vertebrate species, fowls manifest the most noticeable alterations in gonadal size (Dawson *et al*., 2001). Fowls for that matter, have a more complex photoperiodic activities compared to other vertebrate species (Follett *et al*., 1998). Fowls have the most wide gonadal response, despite the short breeding seasons, because the HPG axis is easily closed with an immediate regression of gonads despite the increasing day length, an occurrence referred to as photo refractoriness (Hahn *et al*., 2007; Nicholls *et al*., 1988). The breeding season is shorter in high latitude because of the short mild season in this region. Hamsters and sheep manifest great photoperiodic changes though their seasonal gonad development and regression is less dramatic than among fowls.

Feed availability is the most fundamental requirement that controls the annual trend of reproduction among mammalian species of animals. Reproduction is a high nutrient and energy demanding activity and is therefore evident that mammals are inclined to reproduce during seasons and environments where food availability concurs with season, particularly when food is least available, although either its severe shortage or abundance can result into quick detriment or benefit to reproduction (Bronson & Heideman, 1994). Feed restriction has been reported to have detrimental effect on steroidogenesis than spermatogenesis among male animals, and attainment of puberty, oestrus cycle and pregnancy during late lactation among female animals (Bronson & Heideman 1994). Rabbits experience the effect of temperature in its reproductive activities, whereby, high ambient temperature beyond the thermal neutral zone may have a dramatic effects on their

reproductive physiology, testicular damage and normally results into high embryonic death (Banai & Sod-Moriah, 1976).

The effect of low temperature on reproduction must always be considered alongside feed intake because it demands high feed consumption for high metabolic heat production as an adaptive response to low temperature (Bronson & Heideman, 1994). It has been established that reproductive outcome is one of the aspects in animals that compete for energy and the animal must partition it among the competing demands in the body. A rise in any of these demands must be compensated by increased feed intake, be undertaken at the expense of another demand or the activity is itself terminated altogether (Bronson & Heideman, 1994). The body activities which compete highly with reproduction for nutrients for metabolic heat production include cell maintenance, thermoregulation and locomotive cost related to food finding (Banai & Sod-Moriah 1976). In this competition, reproduction, but more specifically steroidogenesis normally takes the lowest priority and is the first activity to be terminated during moments of energy shortage in the female animal (Bronson & Heideman, 1994).

Among seasonal mammalian species in temperate regions, reproductive activity is restricted to a particular moment of the year to synchronize birth to occur within warm temperature moments and better feed availability which increases the survival chances of the off springs. Previous studies have shown that synchronization of reproductive occurrence to season is controlled by the pineal hormone- melatonin (Ancel *et al*., 2012). Environmental information reaching the pineal gland through the retino–hypothalamo–

pineal pathway generates the release of norepinephrine that acts as a potent and reliable controller of the rhythmic discharge of melatonin from the pineal gland. Melatonin is synthesized and released in a diurnal fashion with increased secretion during night time but reduces to undetectable levels at daytime with the duration of elevated melatonin quantities corresponding to night length (Ancel *et al*., 2012). Among long photoperiod animal breeders, like the Syrian hamster (*Mesocricetus auratus*), an animal that is normally used as a rodent model during the study of seasonal reproductive processes, short day lengths or long nocturnal duration of melattonin discharge inhibits the reproductive system, and any removal of the melatonin hormone signal by pinealectomy prevents this short day inhibition of reproductive activity (Ancel *et al*., 2012). Larger mammals with a longer gestation period, like sheep, are sexually active during short photoperiod and become sexually quiet after transfer to long photoperiod environments (Klosen *et al*., 2013). Although the reproductive timing is opposite in hamsters and sheep, in both cases, the photoperiodic changes in circulating plasma levels of melatonin synchronizes reproductive activities to favorable seasons and is geared for the survival of their off springs.

2.1.5 Hormonal Regulation of the Reproductive System

Hormones regulate reproductive occurrence that involves coordination of multiple organ systems because naturally, they are chemical substances that circulate to most parts of the body. It begins with the hypothalamus integrating environmental and internal cues to ensure synergy and proper timing (Klosen *et al*., 2013). The hypothalamus then releases

gonadotropin releasing hormone, which controls the discharge of the gonadotropin hormones that coordinate reproductive events that include hormone secretion, gonad maturation, development and production of gametes and behavioural cues that ensure successful sexual behavior of the mating mates, gestation and parturition of offspring (Masumoto *et al*., 2010)

Regulation of the reproductive process requires the action of hormones from the hypothalamus, pituitary gland, the adrenal cortex and gonads. At the attainment of puberty in both animal males and females, the hypothalamus secretes gonadotropin-releasing hormone, which causes the secretion and release of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary gland. Follicle Stimulating Hormone stimulates activates the maturation of the ovum inside the follicle. Ones it has matured, the follicles secrete the inhibin hormone to stop any further discharge of FSH from the follicle and this hormones control the gonadal function (Dardente *et al*., 2014). Luteinizing hormone causes the rapture of the follicle for the release of the ovum, causes the secretion of oestrogen and progesterone from the ovaries, the two hormones prepare the animal for gestation. In both males and females, follicle stimulating hormone controls gamete production while luteinizing hormone ensures the secretion of oestrogen and progesterone by the gonads, while elevated discharge of gonadal hormone levels inhibits gonadotropin releasing hormone production through a negative feedback loop (Hazlerigg & Simonneaux 2015; Dardente *et al*., 2014).

The anterior pituitary gland also secretes prolactin in the female animals, which is responsible for the stimulation and milk biosynthesis in the mammary gland immediately after parturition. The posterior pituitary gland secretes oxytocin hormone that is responsible for the contraction of smooth muscles during milk letdown and parturition. The uterine smooth muscles become responsive to oxytocin hormone ones the population of oxytocin receptors attain the peak at the uterus (Dardente *et al*., 2014).

2.1.6 Regulation of the Male Reproductive System

Rabbit bucks are mature for reproductive activity at the age of 32 weeks, at the time of when sperm synthesis has stabilized. The rabbit has high fertility, quite prolific, with a short cycle of reproduction, which marks onset of reproductive cycle at marks the 4 – 5^{th} month among light breeds (Onuoha, 2020). Regulation of the male reproductive system starts with gonadotropin releasing hormone when it stimulates the synthesis and secretion of follicle stimulating hormone and luteinizing hormone. The processes are determined by size and frequency of gonadotropin releasing hormone pulses, and feedbacks from androgens (Dardente *et al*., 2014). A Low-frequency gonadotropin releasing hormone pulse brings about follicle stimulating hormone release, but high-frequency pulses bring about luteinizing hormone secretion. Follicle stimulating hormone causes the first sub-division during meiosis in spermatocytes to develop into secondary spermatocytes, and eventually lead to their development into mature sperm cells (Petrulis, 2013).

Luteinizing hormone stimulates the secretion of testosterone by the cells of Leydig in the testes, which enhances the biosynthesis of sperm and development of male masculine characteristics.

Upon attaining the optimum level of spermatozoa, a negative feedback mechanism takes place in the male to curtail further rise of sperms by action of testosterone on the hypothalamus and anterior pituitary to inhibit further secretion of gonadotropin releasing hormone, follicle stimulating hormone and luteinizing hormone. The Sertoli cells on their part secretes inhibin hormone into plasma particularly when the sperm count is too high. Inhibin hormone slows down spermatogenesis (Dardente *et al*., 2014).

High libido among male animals is an important factor in reproduction but is under the influence of a number of factors that include nutritional status-particularly cholesterol, health, age, testosterone level and social behavior within the group (Swelun *et al*., 2017).

2.1.7 Regulation of the Female Reproductive System

The regulation of the reproductive system in female rabbit commences at the age of 32 weeks. Rabbits are highly fertile, quite prolific, with a short cycle, which marks onset of reproduction among light breeds (Onuoha, 2020). This normally starts at age of 4 – 5th month of age and since then, the reproduction life is controlled by hormones. The follicle stimulating hormone controls the development of ova cells in follicles. Follicular cells also synthesis the hormone inhibin that inhibits the follicle stimulating hormone production in

the system ones it is not required, while luteinizing hormone affect the developments of the ova, causes ovulation, and synthesis of oestradiol and progesterone in the ovaries (Hazlerigg & Simonneaux, 2015). These two hormones; oestradiol and progesterone are important for the preparation of the uterus for the attachment and maintenance of pregnancy, while oestradiol brings about the development of female secondary sex characteristics, and both oestradiol and progesterone control and regulate the menstrual cycle in women (Hazlerigg & Simonneaux, 2015).

The posterior pituitary gland secretes oxytocin hormone that is responsible for uterine contractions during birth. The uterine muscles are not responsive to oxytocin hormone until late during pregnancy, this is the moment oxytocin receptors in the uterus will have reached the peak, the tissues in the uterus and cervix are stretched and this stimulates oxytocin discharge during birth. The intensity of the contractions increases when the blood levels of oxytocin peak via a positive feedback mechanism until when the birth process is complete (Petrulis, 2013). Oxytocin causes contraction also, of the myo-epithelial cells surrounding the milk-producing mammary glands to force milk from the secretory alveoli into milk ducts from where it is ejected in a reflex action for harvest by the young animal, which is responsible for its stimulation by the suckling of the teats, an action which triggers further synthesis of oxytocin in the hypothalamus and its release into circulation at the posterior pituitary (Petrulis, 2013).

The anterior pituitary gland controls body endocrine functions, which sends signals to specific glands to affect activities that include fertility (Norton, 2007). Damage (trauma,

infection, cancer or radio therapy) on the anterior pituitary gland may result into hormonal deficiency, where it may not release the specific hormone required for a given function (Norton, 2007). The anterior pituitary gland is responsible for producing follicle stimulating hormone and luteinizing hormone which are responsible for oestradiol production and follicle maturation in females (Dardente *et al*., 2014).

The female gonad produces oestradiol that regulates and integrates the functional activity of the reproductive system and gametes for eventual fertilization and formation of the embryo. The female reproductive processes are more intense and demand more energy expenditure than males of the same specie as the female is preparing for extra tissue mass after conception (Pineda, 1989). According to this author, the reproductive activities of the female of any species are the first to be arrested when the animal is confronted by debilitating illness, nutritional deficiencies or life threatening disease. Likewise, conception is only the beginning of reproduction for the female, as it must go through gestation and the birth of an offspring. The uterine environment therefore, must be healthy for gamete encounter and fertilization, embryonic development, attachment, gestation and successful completion of pregnancy (Pineda, 1989).

2.2 Female Reproductive cycle and fertility

Reproductive cycles are known to occur either oestrual or menstrual types, named in order to be used for external characterization for particular stages of the cycle and in relation to

endocrine action and time of ovulation (Brockington, 2017). Oestrus cycle has stages based on ovarian developmental stages in major mammals as shown in figure 2.1

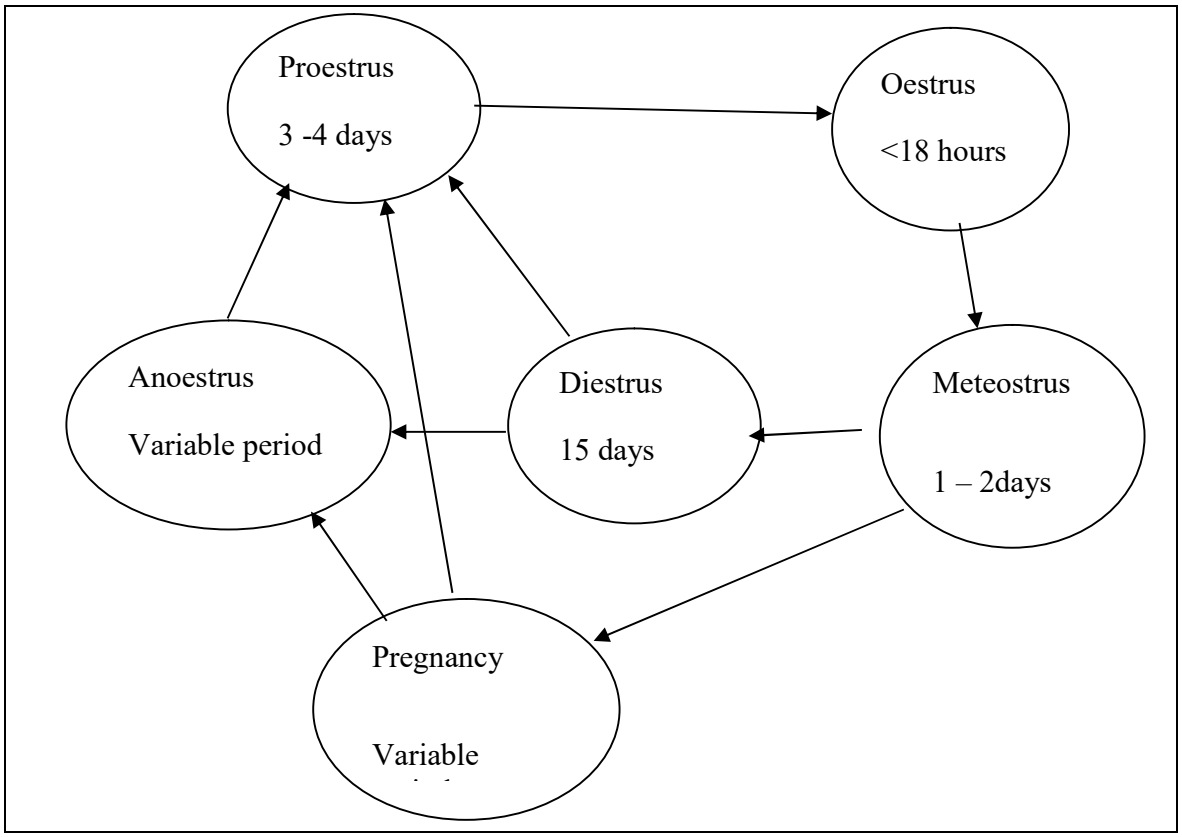


Figure 2.1: Stages of Ovarian cycle in mammals (21 days oestrus cycle) (McDonald, 1989)

The reproductive cycle among mammals is influenced by a number of factors, both within the animal itself and others from the external environment it lives in. These factors include attainment of puberty and sexual activity, season of breeding sexual activity during postpartum period, but most of which are regulated by the genetic constitution of the

individual animal, its environment, endocrine system and the psychosocial factors in the anima's surrounding (Hafez & Hafez, 2000).

One's female mammals are at point of completing their reproductive cycle, they advertise their readiness for mating through signals in urine that contain metabolic products of various hormones and secretions of reproductive organs which signal the female's reproductive state that attracts the male partner for copulation (Johnson, 1983).

Mammalian animal species display periodic breeding patterns that correlate to short or annual cycles which are indications of changes in physiology and morphology of the reproductive system; some of these cycles are photoperiodically controlled (Elliots, 2006). The circadian time measuring process depends on the oscillation of responsiveness to light and suggests the participation of the pineal gland and the suprachiasmatic nucleus in the photoperiodic regulation of mammalian reproductive cycle (Elliots, 2006). Through the secretion of melatonin hormone, this environmental factor is incorporated into the annual cyclic reproductive activities of certain species of animals.

Nutritional status and energy reserves are necessary for optimum hypothalamic – pituitary-gonadal function and has great impact on reproduction, regulation of seasonal reproductive behaviour and is the main cue controlling fertility in cycling animals (Bova *et al*., 2014). It has beneficial effects on reproduction through multi-hormonal controls, particularly the gonadotropic and metabolic hormones (Abadjieva *et al*., 2011). Lassoued *et al*. (2004) showed important interactions between genotype and level of nutrition, where higher level

of nutrition prior to and during mating was associated with improved reproduction. An optimum nutritional status and energy reserve are necessary for hypothalamic – pituitary – gonadal integrity and has great influence on endocrine systems, where the beneficial effects are seen through multi-hormonal actions which include gonadotropic and metabolic hormones (Bova *et al*., 2014; Abadjieva *et al*., 2011). A functional defect on any of the components of this hormonal complex, affects reproduction directly (Petrovic *et al*., 2012). Body reserves and feed intake contribute nutritional feedback to the hypothalamus, although reproductive neuroendocrine output from GnRH/LH, is more stimulated by feed intake than by adipose tissue (Petrovic *et al*., 2012; Forcada *et al*., 2006).

Meza-Herrera & Tena–Sempere (2006) reported that that nutrient supplementation for 4 – 6 days at pre-ovulation increases an animal's ovulation rate by 20 – 30% without detectable change in body weight or body condition.

The control of the hypothalamo-pituitary-gonadal axis includes hormone regulation, neuron and glial cells that facilitate reproduction (Christensen *et al*., 2012). The neuro-endocrine regulation results into a single product release of gonadotropin releasing hormone, which is also influenced by coordination of internal and external factors. The effect of each environmental signal varies among organisms where some are highly responsive to day length, fluctuation in temperature, olfactory input, nutrient availability, light or acoustic stimuli and internal signals such as steroid hormone feedback and hormone controls (Christensen *et al.,* 2012). Both external and internal cues converge to regulate the GnRH

generator for transduction of the cues into reproductive output according to Christensen *et al*. (2012).

Suckling, as a factor that can alter cyclic reproductive activities, stimulates the hypothalamus to secrete oxytocin and prolactin, and both prolactin and suckling, inhibit the pulse centre of GnRH, which thus suppress LH and FSH secretion (Medina & Mejia, 2019) The reproductive cycle is an expression of the synchronized action that brings about interactions between the hypothalamic – pituitary – gonadal hormonal systems. The aims of the cycle are to bring to maturity follicles having ova that end up in ovulation and fertilization, bring about structural and functional changes in uterus, oviduct and vagina to facilitate fertilization and implantation of conceptus and to facilitate early pregnancy (Bronson, 1989). The activities of the cycle takes place in three phases; follicular phase when oocytes prepare and attain ovulation stage, the ovulatory stage when the ovum is released from ovaries and finally, the luteal stage when the ova may come in contact with sperm and pregnancy takes place (Bowman & Miller, 2001).

The functions of the ovary include production of female gamete and to support the developing gamete by steroidogenesis. The dominant hormones among the mammals like human and primate females are gonadotropin releasing hormone from the hypothalamus; luteinizing and follicle stimulating hormone produced by the anterior pituitary gland and oestradiol, inhibin and progesterone from ovaries. The duration of phases and the length of

the cycles vary according to the mammalian species, but the activities that occur in the ovaries are apparently the same (Baum & Cherry, 2015).

Animal reproductive cycle depends upon interrelationships between sex hormones and gonadotropic hormones. Under optimum conditions, follicle stimulating hormone, with the support of luteinizing hormone, promotes growth and the secretory activity of the follicles, to release oestrogen. Increased output of oestrogen, causes reduction of FSH output, for the purpose of controlling follicular growth, but stimulates the secretion of LH, for the promotion and development of corpus luteum, whose role is the maintenance of pregnancy. A defect in any of the components of the reproductive hormone complex directly affects the reproductive cycle (Patrovic *et al*., 2012).

2.3 Morphology and Cell apoptosis of follicles

The rabbit (*Oryctolagus cuniculis*) has small ovaries, whose shapes are flat ovoid organs, located at both lateral pelvic cavities. Surfaces of *Oryctolagus cuniculis* ovaries are protected by a layer of epithelial cells. A large membrane called *tunica albuginea*, divides the cells at the surface from where it separates the ovary tissue into the medulla on the inside and outer cortex, which contains the follicles and stroma. The mammal's ovarian follicular cell layers forms the wall of the compound epithelium, which contains a very small potential difference across the follicle wall (Makarevich *et al*., 2002).Follicular population in mammals of major and low ovulation rates have been studied and established that animals with higher follicular number at each point over the entire development phase

has greater ovulation rate (Danko, 1997). The number of follicles at a particular stage at any moment dependents on the population of follicles within the growth phase and the rate at which these follicles go through the phase (Ariyaratna & Gunawardana, 1997; De Bruin *et al*., 2001).

The reported differences among the population of follicles have been established to be possible channels for evolving genetic effects in ovulation rate (De Bruin *et al*., 2001). Gonadotropin hormones and ovarian steroid hormones control the proliferation and differentiation of granulosa cells in developing follicles (Fukumatsu *et al*., 1992; Maraček *et al*., 2002). The paracrine and autocrine intra ovarian functions of the development of follicles are also effected, while the epidermal and fibroblast growth factors cause the growth of granulosa cells (Makarevich *et al*., 2002).

It is hypothesized that the foetal source, growth and development of the organs at the abdomen is interfered with by intra-uterine developmental restrictions, resulting into disease outcome after wards in life. The sizes of animal ovaries improves substantially from the second half of gestation. The average population of ovarian follicles and the average follicular diameter substantially increase on weekly basis by 0.48% and 0.52 Im, accordingly. Intermediary follicles (72%) form the majority, while Primordial follicles reduce from over 20% to below 10%, while the primary follicles rise in number from 6 to 19%. Oogonia are frequently present before the 30th week of gestation among cattle (De Bruin *et al*., 2001).

The mammalian adult ovary carries a reservoir of dormant primordial follicles, which have a tiny non-developing oocyte plus a group of non-proliferating pre-granulosa cells held by the follicular basal lamina. On a daily basis, a number of primordial follicles are activated, and the oocyte starts developing while the granulosa cells start sub-dividing. The sub-division of the granulosa cells, the membrana granulosa or follicular epithelium about the oocyte multiply and the follicular basal lamina increases in size. After some time of development, a fluid-filled antrum expands and specialized stroma cell layers, the theca interna and externa, proliferate. The follicles that attain the stage of the expanded large antrum, and at the follicular wave after the regression of corpora lutea, is expected to ovulate an oocyte in reaction to the surge wave of Luteinizing Hormone (Rodgers & Irving Rodgers, 2009).

The Preantral follicles of bovine and human beings can be categorized into two groups based on the morphological characteristic of the follicular basal lamina (Irving-Rodgers & Rodgers, 2000; Irving-Rodgers *et al*., 2008). In both categories, follicles contain a conventional basal lamina with a single layer assigned to the surface of the granulosa cells, these at pre-antral stage in all categories are emphatically thick or in partial lamination compared to the primordial or the antral follicles. Pre-antral follicles containing more layers of basal lamina have been noticed, while loops of basal lamina are frequently noticed in parts and are joined to more layers nearer to the granulosa cell surface. Cellular protrusions emerging from the outside of the basal granulosa cells and vesicles covered by

membranes are commonly at the edge are present and adjacent to the basal lamina (Irving-Rodgers & Rodgers, 2000).

The idea of categorized control of granulosa cell multiplication and follicular liquid containment has been useful in explaining two follicular phenotypes (Rodgers *et al*., 2001; Irving-Rodgers & Rodgers, 2000). These categories among bovines can be separated based on morphological and activity criteria. The first phenotype show the 'loopy' basal lamina while the other manifests the columnar basal granulosa cells and manifestation of the RNA unit of telomerase (TERC) in the antral located cells (Irving-Rodgers & Rodgers, 2000; Lavranos *et al*., 1999) and discussed in Rodgers *et al*., (2001).

It was hypothesized that in the event that the follicular antrum expansion is at a low rate compared to the replication of the granulosa cells, the cell layers become crowded resulting into more of the basal layers of cells lading columnar shape (Rodgers *et al*., 2001). The concept connecting basal granulosa cell shape and follicular basal lamina phenotype to the speed of follicular antrum growth agreed with the finding that columnar basal granulosa cells and loopy basal laminas are not found in follicles that have grown or beyond the dominance size or where the acceleration of antrum growth is reported to be dominant by ultrasonography (Irving-Rodgers & Rodgers 2000; Irving-Rodgers *et al*., 2002).

Report has it that follicles containing loopy follicular basal lamina manifest slower growth rate, but produce more basal lamina that is eventually discarded from the surface of the granulosa cells by discarding those parts of the outer membrane that has focal adhesions

and which produce the visible membrane-bound vesicles as observed from the discard of fibroblasts from collagen matrix (Rodgers *et al*., 2001; Lee *et al*., 1993). Reports that the oocyte quality may be poor could stem from the theory that the follicles may be slow growing and therefore 'older' in terms of the length of time since its invigoration.

It is also possible that oocytes obtained from follicles containing loopy follicular basal lamina may not appropriately stimulate multiplication of granulosa cells or antrum expansion resulting into the reported loopy follicular basal lamina phenotype. Implying then that follicles containing a loopy follicular basal lamina may be an indication of an early case of atresia before any observable evidence of cell death. Since the oocytes of older women are of poorer quality and since in cows follicle numbers in a wave decline with age, it would be interesting to know whether such ovaries contain an increased proportion of follicles with loopy basal lamina. (Broekmans *et al*., 2009; Malhi *et al*., 2005)

2.3.1 Follicular Cell atresia

Atresia of follicles is a major loss of the entire follicle and not mare death of an individual single cell, though even the death of one cell, like an oocyte, is a big loss despite how early it may occur in the process. Cell atresia has been theorized to be a normal part of tissue homoeostatis. The main focus of studies on this topic is often on cell death, but atresia is also an active natural cellular activity and could involve resorption of the follicle with the action of macrophage infiltration, phagocytosis, migration of fibroblasts from the theca and

production of collagen, which are similar to the processes normally occurring in wound healing (Martin 1997, Schultz & Wysocki 2009). A number of methods have been applied to categorize follicular atresia, despite their classification into healthy and atretic, using such methods that include histology, biochemical markers and ultrasonagraphy; and as there may exist no agreement on the most acceptable method for classification of atresia, the use of the several different ways has leads to different phenotypic categorizing of follicles from one method to another.

Primordial follicles among most animal species are observed and categorized histologically, mainly based on their small non-growing oocyte, lacking a zona pellucida but covered by flat granulosa cells (Fair et al., 1997). The follicles appear so as the oocyte is not growing while the granulosa cells are not multiplying. Once activated, the primary follicles have a growing oocyte immediately they are activated with the zona pellucida around it having been formed, and an arrangement of granulosa cells showing cuboidal shape (Fair *et al.*, 1997). The cuboid shape is an indication of growth and commencement of replication and cells 'round-up' at the prophase and metaphase allowing spindle development and sub-division to follow (Boucrot & Kirchhausen 2008; Rosenblatt 2008). It has been confirmed that in the mouse ovary, the cuboidal granulosa cells sub-divide effectively compared to flat cells (Da Silva-Buttkus *et al*., 2008).

During follicle growth, a number of cells could differ in their vulnerability to death and if such cells are not replaced then it will result in follicular atresia. During bovine preantral

period, oocytes have been observed to be the first to die, as compared to the antral time when granulosa cells die first, and in one form of atresia thecal cells, involving steroidogenic and endothelial cells, also die very early in atresia(Rajakoski 1960; Clark *et al*., 2004). Loss of growth factor support such as TGFa or that expression of Fas or Fas ligand initiates a large number of cell death could be involved in initiating the process (Wang *et al*., 2002; Porter *et al*., 2001, Quirk *et al.*, 2004). But the death of one cell, like the oocyte, ones it is involved in the commencement of atresia, is likely to involve the failure in the proliferation of the oocyte, but failure of a small number of granulosa cells may not produce the same results. A limited number of death of granulosa or thecal cells would be expected in a healthy follicle, this contributes to the hardship to define accurately the commencement of follicular atresia and the determination of the required level of cell death to indicate atresia (Jolly *et al.*, 1994).

Studies of bovine follicles have determined steroid hormone amount in follicular fluids with varying manifestations of atresia or cell death to be used to ascertain whether the steroid amount are in any way good indicators of atresia (Ireland & Roche 1983, McNatty *et al*., 2006; Grimes *et al*., 1987; Jolly *et al*., 1994). The agreement arrived at was that healthy follicles have higher levels of oestrogen than atretic follicles, while atretic follicles were found to have more quantities of progesterone or theca products like testosterone or androstenedione for the same size of follicle. The terms ‘oestrogen active’ or ‘inactive’ are often used to mean either healthy or atretic follicles respectively. But, the absolute

quantities of progesterone, androstenedione and oestrogen go up with increasing follicle size up until ovulation, and hence absolute levels can only be used as a basis of comparison between similar sized follicles. To overcome this problem, ratios of oestrogen to progesterone or testosterone were employed and it indicated a decrease substantially on atresia (Ireland & Roche, 1983).

2.4 Biological Characteristics of New Zealand white rabbit

The rabbit *(Oryctolagus cunniculus*) is a pendadactyl-herbivorus mammal, classified midway between a ruminant and monogastric. It exhibits unique feeding habits and belongs to the order Lagomorpha and leporidae family (Brun & Ouhayoun, 1989). Initially rabbits were kept as pet animals but man has domesticated them for commercial purposes and as a source of protein, generally referred to as white meat, due to its low fat content and therefore of less risk of causing heart diseases to human beings. Rabbit production is new and is a small-holder enterprise that has advantages over the other livestock systems due to its small body size, high rate of reproduction, adaptability to inexpensive housing and production of useful by-products (Owen *et al.,* 1997).

They are generally multipurpose animals used for meat, fur, wool and laboratory experiments. Its wool is lighter and warmer than that of any other animal. Its white meat has the benefit of having low cholesterol levels for people with health and nutritional restrictions and any one with greater awareness of the need to control life style diseases through dietary means. After slaughter 93% of the entire carcass is consumable because,

about 7% of the rabbit consists of bone. Rabbits have so far no known communicable diseases to human beings but instead have been reported to be helpful in stomach indigestion of food (Romney 1981).

New Zealand white rabbit weigh 2 -6 kg, with a variable lifespan of 5 – 6 years. It has a respiratory and heart rate of 32 – 60 and 130 – 325 breaths and beats per minute respectively. New Zealand white has a normal rectal temperature of 38.8^{o}C; while all its teeth are open rooted and grow continuously.

Rabbit caecum is large, thinned walled, coiled and terminates at the caecal appendix. The appendix contains a large amount of lymphoid tissue for the promotion of the rabbit's immunity. The other organs in its body are quite developed to function properly for the survival of the rabbit. The left and right lungs have two and four lobes respectively. The right antrioventricular valve of the heart, tricuspid valve, has two cusps. The function of the valve is to prevent any back flow of blood from the right ventricle into the right atrium.

The onset of Breeding for the medium size breeds, of which New Zealand white is one, starts at the age of 5 – 6 months for the doe but one month latter for the buck. The does are induced ovulators, whereby, the ova are released 10 – 13 hours following copulation. Ones the buck is paired to the doe, mating will occur in about 30 minute's time. Gestation period takes an average of 32 days and a doe will kiddle to 7 – 8 kittens in its litter. A few days to kidding, the doe will start to prepare the nest by removing hair from her legs and belly for purposes of covering the kids. The kittens are hairless and weigh 30 – 100 g. The kitten

will have their eyes opened at about 10 to 12 days after kiddling (http://ivma.org/educational-material/biology-of-the-rabbit.aspx).

2.5 Mycotoxins as food contaminants

2.5.1 Distribution of Mycotoxins

Mycotoxins have alarmingly become important global problem in relation to feed /food safety and poisoning. They are abiotic substances secreted by fungi that have been known to grow on various crop species. The most common Mycotoxins in food stuffs include ochratoxin, deoxynivalenol, fumonisins, zearalenone and aflatoxins. The moulds which produce mycotoxins in foodstuffs are categorized into pre-harvest invaders, referred to as field fungi, and those found strictly post-harvest, also referred to as storage fungi. The toxic pre-harvest fungi include Fusarium *graminearum,* which thrive on stressed plants, and these include *Fusarium moniliforme* and at times *Aspergillus flavus* (Assefa *et al.,* 2012).
The others are initial colonizers of plants before harvest, which predispose the crop to mycotoxin contamination post-harvest or in storage and these include; *Penicillium verrucosum* and *Aspergilus flavus*.

Mycotoxin concentration in industrial by products is normally low compared to raw materials (Marin *et al*., 2013, Ahmed and Jutta, 2015). Mycotoxins are also referred to as substance of low molecular weight secreted by moulds that elicit a toxic reaction by natural means of exposure both in humans and vertebrate animals (Zain, 2011). The predisposing factors include physical variables such as environmental conditions suitable to fungal

colonization and mycotoxin production such as temperature, relative humidity and insect infestation. Climatic factors for mycotoxins production include poor hygienic conditions during transport and storage, high temperature and high moisture content or heavy rains. Chemical factors at work are the fungicides or fertilizers, while favourable biological conditions are determined by the interaction between the colonizing toxic fungus and their substrate (Assefa *et al.,* 2012).

Mycotoxins infest several food products including animal feedstuff, cereal crops, leguminous plants and animal products. High density animal feed stuffs, particular those containing sorghum are in most cases with high levels of Aflatoxin. Health effects occur in companion animals, livestock, poultry and humans because aflatoxins are potent hepatotoxins, immunosuppressant, mutagens and carcinogens *(*Assefa *et al.*, 2012).

Mycotoxins are produced in temperate, sub – tropical and tropical climates, whenever rainfall and humidity are experienced during the harvest season (WHO, 2005). Aflatoxin contamination is promoted by stress or damage to the crop due to drought prior to harvest and inadequate drying during storage (Sasaki *et al*., 2002; Turner *et al*., 2005). Fungi are extremely adaptable organisms with the capacity to metabolize a large variety of substrates over a wide range of environmental conditions and are produced only under aerobic conditions (Cardwell & Cotty, 2002). These authors reported that crop contamination by aflatoxin occurs at environments between approximately 40^{o}North and 40^{o} south of the

equator, particularly within developing countries, due probably to physiological adaptation to climatic regime for optimum enzymatic action for the survival of the fungi.

Mycotoxins occur naturally in a wide variety of feedstuffs used in animal feeds. In most European countries aflatoxins are not quite prevalent but vomitoxin, ochratoxin and zearabenone (Akande *et al*., 2006) are prevalent because they are favoured by temperate conditions. Aflatoxin is common in humid climatic conditions similar to those in Asian, Africa and Australia with *Aspergillus* genera being predominant in these regions. These moulds invade seeds and grains before harvest or under moist storage conditions.

Table 2.1: Geographical distribution of different genera of mycotoxins

Location	Mycotoxin
Western Europe	Ochratoxin, Vomitoxin, Zearalenone
Eastern Europe	Zearalenone, Vomitoxin
North America	Ochratoxin,Vomitoxin, Zearalenone,aflatoxin
South America	Aflatoxin, fumonisins, chratoxin,Vomitoxin,T-2 toxin
Africa	Aflatoxin, fumonisins, Zearalenone
Asia	Aflatoxin
Australia	Aflatoxin, fumonisins

(Source: Devegowda *et al.*, 1998)

Mycotoxins are regularly found in feed ingredients that include maize, sorghum, barley, wheat, rice meal, groundnut and some legume plant species.

The dominant mycotoxin in the tropical and sub-tropical humid regions is the aflatoxins. It is reported to be high during rainy season as compared to dry seasons according Martinez & Blasco (2015). Aflatoxins are also higher in wet and dry total mixed rations for dairy cattle than dry feeds and concentrates.

The problem of aflatoxin contamination in food, feed and grain by-products is prevalent in tropical and sub-tropical regions especially in sub-saharan countries due to poor field handling of and storage practices, high prevailing temperature and humidity (Bennet *et al*.,

2003). They can contaminate feedstuffs at any point from field to stores and some feeds that include grains, feed by- products, protein concentrates and finished feeds, oilseed cakes and forages are more prone to contamination (Waldner &Lalman, 2010).

2.5.2 Chemical structure and physical properties of aflatoxin

Table 2.2. Chemical and physical properties of aflatoxins

Aflatoxin	Molecular formular	Molecular weight	Melting point
B_1	$C_{17}H_{12}O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O_7$	330	237-240
M_1	$C_{17}H_{12}O_7$	328	299
M_2	$C_{17}H_{14}O_7$	330	293
B_{2A}	$C_{17}H_{14}O_7$	330	240
G_{2A}	$C_{17}H_{14}O_8$	346	190

(Source: Reddy and Waliyar 2000)

Aflatoxin are metabolites produced by fungi in feeds and foods and the major ones, occur in forms that include: aflatoxin B1, B2, G1, G2 and M1, with the latter type being formed from B1 in both animal serum and milk (Chase *et al*., 2013).

The chemical structure of the common aflatoxin types are shown in figure 2.2.

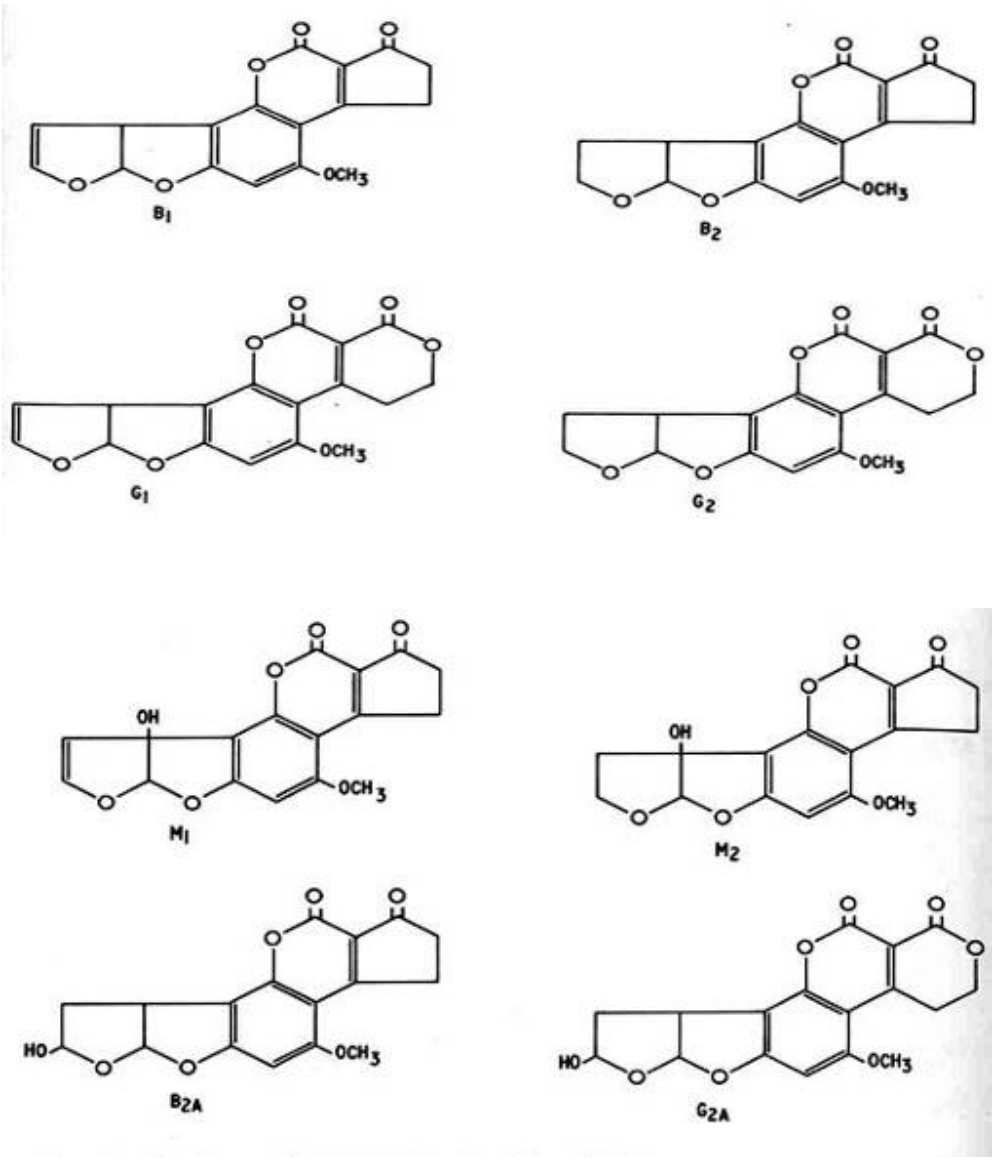


Figure 2.2: Structures of aflatoxins B1, B2, G1, G2, M1, M2, B2A and G2A (Source: Reddy and Waliyar 2000)

Aflatoxin B1 is the most toxic mycotoxin among the lethal carcinogens (WHO, 2005). Aflatoxins M1, M2, B2A, and G2A which may be produced in minor quantities have been isolated from cultures of *Aflatoxin flavus* and *Aflatoxin parasiticus*. A number of closely related compounds namely aflatoxin GM1, parasiticol and aflatoxicol are also produced by *Aflatoxin flavus*. Aflatoxin M1 and M2 are metabolites of aflatoxin B1 and B2 respectively, found in milk of animals that have consumed feed contaminated with aflatoxins B1 and B2 (Reddy & Waliyar, 2000). The acceptable levels of aflatoxin in human food and cattle feed is given in Table 2.2

Table 2.3: Aflatoxin allowable levels and sources for human and animal food.

Animal Species	Aflatoxin levels (ppb)	Feed source
Human	0.5	Milk
Human	20	Grain sources
Rabbit	50	Grain sources (concentrates)
Dairy & Stressed cattle	20	Corn, peanut products, cottonseed meal, other animal feeds and feed ingredients intended for animal species or uses not specified above, or when the intended use is not known
Immature cattle	20	Corn, peanut products, and other animal feeds and feed ingredients, but excluding cottonseed meal
Breeding Swine, poultry	100	Corn and peanut products
Finishing swine	200	Corn or peanut
Beef cattle (breeding)	300	Corn and peanut products
Feedlot cattle	300	Corn and peanut products

(Source: FDA, 2011 & FAO, 1997)

The biological effects of aflatoxin depend on the ingested amounts, type of toxin, duration of exposure and animal sensitivity (Akande *et al*., 2006). The variations of recommended amounts shown in table 2.2 occurs because non lactating mature animals take less

concentrates but more forage, which in turn reduce the amount of aflatoxin in their diet (Waldner & Lalman 2010) and the metabolic reactions that take place during the process are product dependent influencing the level of aflatoxin hydroxylation and detoxification. Balaraman & Arora (1986) found that calves consuming 0.5, 1.0 and 1.5 ppm of aflatoxin in dry matter feed having reduced weight gains with increasing levels of the aflatoxin in their diet, although their feed intake was not significantly affected. These authors found calves consuming diets with more than 1.0ppm of aflatoxin not being able to recover to full potential performance even 16 weeks following the removal of aflatoxin from their diet. The permitted limits of aflatoxin B1 in foodstuffs range from 0 to 30 µg/kg, while those for all the species of aflatoxin (B1, B2, G1, G2 among others) range from 0 to 50 ppb (FAO, 1997), based on their lethal doses.

Aflatoxicosis in both human and animals occur either in the acute form or in chronic form. The acute form normally occurs upon consumption of moderate to high levels of aflatoxin over a short time. This acute form can be observed when symptoms such as hemorrhage, liver damage and disturbance of nutrient digestion, absorption or metabolism occur (Barrett 2005).

The second form is the chronic aflatoxicosis which occurs following prolonged intake of low to moderate amounts of aflatoxin and the effects are usually subclinical and difficult to recognize. Chronic aflatoxicosis could cause impaired feed utilization, teratogenic effects or gene mutation (Thrasher *et al*., 2012; Wangikar, 2005). The nature of gene mutation is

brought about by aflatoxin B1, which reacts with the gene to form, adducts. Aflatoxin B1 exo-8, 9-epoxide reacts covalently with DNA to form adduct that account for the biological effects of aflatoxin, with the most common adduct being 8, 9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxinB1. Another most frequent mutation induced by aflatoxin is the GCTA Trans version (Smela *et al*., 2001).

2.5.3 Aflatoxin absorption and Mechanism of action

Aflatoxin strain toxicity is variable according to the different chemical structure, sensitivity of the species of toxin and animal related factors such as sex, age, health and its diet. They are highly liposoluble compounds that are readily absorbed through the gastrointestinal and respiratory tract into the blood stream (Agag, 2004). Human beings and animals get exposed to aflatoxin through direct ingestion of contaminated food or the moulds carried over from feed into milk and other animal tissues in form of AfM1 (Agag, 2004) or by inhalation of dust particles of AfB1 in foods in industries and factories.

Aflatoxins are absorbed across the gastrointestinal mucosal cell membranes into blood circulation from where they are distributed to tissues and to the liver. The liver metabolizes aflatoxin into reactive expoxide intermediates or they are hydroxylated to become less harmful aflatoxin products. In humans and other susceptible animal species, they are metabolized by cytochrome P 450 (CYP 450) enzymes to aflatoxin – 8, 9 expoxide a reactive form that binds to DNA and albumin in the blood serum causing DNA damage (Wild Montesano, 2009; Wu & khlangwiset, 2010).

Cytochrome Phosphate 450 enzyme isoforms occur in the liver and they metabolize aflatoxin into aflatoxin 8, 9 – epoxides which may bind to proteins and cause acute toxicity (aflatoxicosis) or to DNA and induce liver cancer (Wu & Khlangwiset, 2010). CYPIA2 in a detoxification pathway is capable of catalyzing epoxidation and hydroxylation of AfB1 to form aflatoxin M_1 (Guengerich, 1998), this concurred with Wogan and Pong (1974) who reported that hydroxylation of B1 and B2 resulted in the formation of sub-types M1 and M2 respectively.

2.5.4 Metabolism of aflatoxin

Aflatoxin is basically metabolized by cytochrome P450 enzymes in the liver, but can also occur at the site of absorption, in the blood or in several extra-hepatic organs. Several enzymes belonging to CYP450 family metabolize aflatoxin by oxidative reactions converting them into metabolites with different harmful potentials. The involvement of a wide variety of CP450 isozymes from several gene families in the metabolism of aflatoxin identified as P450IA, P450IIB, P450IIC, P450IIIA and P450IVB (Forester *et al*., 1990). The majority of the activities of cytochrome P450s are deactivation less toxic metabolites by ketoreduction or aflatoxinM1 by hydroxylation or production of non-toxic metabolites including afB2a or afQ1 by way of hydroxylation, or the formation of afP1 by demethylation (Deng *et al*., 2018). But others like epoxide hydrolase are bio activation, convert aflatoxin into reactive products that modify cellular constituents and cause serious damage to cellular organs (Guengerich, 2006). The family involved in bio-activation of

afB1 according Guengerich, (2006) is cytochrome P450IIA, but in combination with other enzyme species as reported by Forester *et al*., (1990).

The possible metabolic pathways of aflatoxin can be through bio-activation, conjugation or de-conjugation into other molecules such as aflatoxin P1, aflatoxicols, aflatoxin M1 or the acutely lethal AfB1-8,9-epoxide. Aflatoxin B1 could also be converted into less toxic compounds such as AfQ1 or AfB2. The pathway is determined by the cytochrome P450 enzyme family as detected by the genetic predisposition of the consuming animal, age and nutritional status (Howard *et al*., 1990). Different forms of cytochrome P450 serve different transformation functions depending on animal species. The main CYP450 being enzyme CYP3A4 and CYP1A2, which are isozymes that are mainly active in the liver (Wu *et al*., 2009). These enzymes with others in the liver determine the degradation pathway whether it is epoxidation, hydroxylation, hydration or demethylation. The product of metabolic processes is determined by the reaction as shown in figure 2.3.

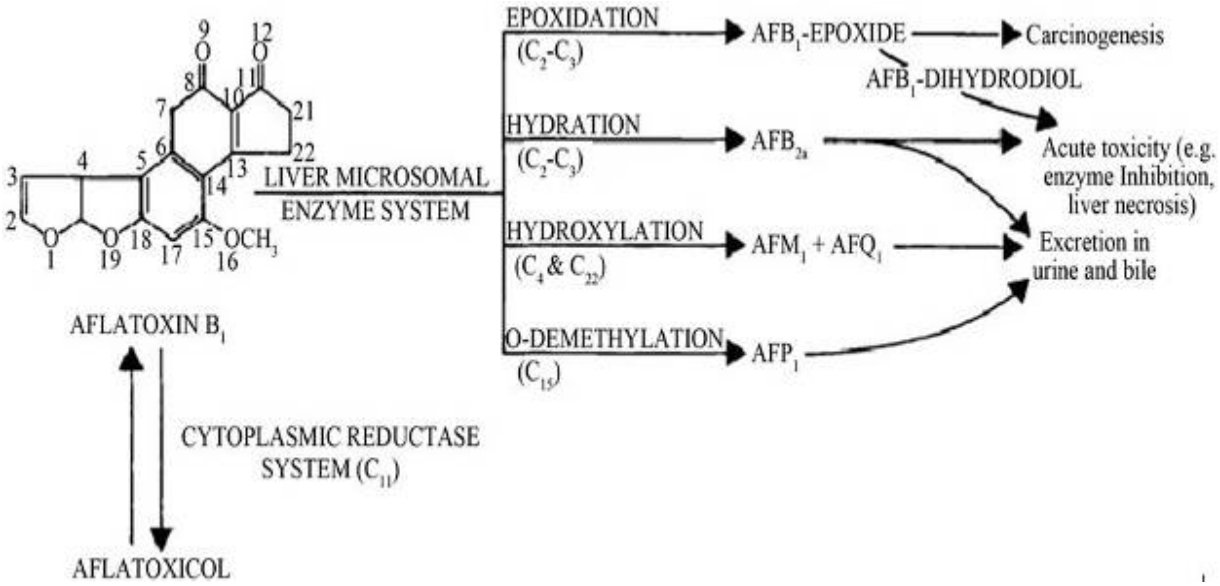


Figure 2.3: Pathways determining toxicity of aflatoxin
(Source: Dhanasekara *et al*., 2011)

The toxic effects of aflatoxin in the liver are linked to their activation into free radicals AfB1-exo-8,9 – epoxide by cytochrome P450 enzymes and associated formation of reactive oxygen species (Saad-Hussein *et al*., 2019).

Studies have shown increased levels of aflatoxin in milk within 12-24 hours after animals consume feeds with high quantities of the aflatoxin, but can be removed from milk within 1-4 days when it is omitted from the ration. The aflatoxin in milk is the M1 form, which is reported to be 1-3% of the aflatoxin consumed in feed in form of AfB1 (chase *et al*., 2013). Lactating animals consuming feed containing 20 ppb of aflatoxin, the permitted quantity, will have less than 0.1ppb of aflatoxin M1 in milk, which is less than the accepted quantity of 0.5 ppb in milk (Waldner & Lalman, 2010).

2.5.5 Aflatoxin Pathogenicity

Aflatoxin in the sub-Saharan region is produced by *Aspergillus parasiticus* and *Aspergillus flavus.* The four major aflatoxin strains produced by Aspergillus species of moulds include aflatoxin B1 (AfB1), aflatoxin B2 (AfB2), aflatoxin G1 (AfG1) and aflatoxin G2 AfG2. The "B" and "G" refer to the blue and green fluorescent colours produced under Ultra-violet light on a thin layer chromatography plates, while numbers 1 and 2 indicate **major** and **minor** compounds respectively (Bennett *et al*., 2003; Thrasher, 2012; WHO, 2005; Sudakin, 2003) which are indicators of their toxicity. *Aspergillus flavus* produces aflatoxin B_1 and B_2; while *Aspergillus parasiticus* produce aflatoxin B1, B2, G1 and G2 (Fratamico, 2008). The metabolic products of aflatoxin B1, which are afM1 and afM2 are found in milk of lactating and blood among animals fed on mouldy feeds (Bennett *et al*., 2003).

Among the four major aflatoxins (AfB1, AfB2, AfG1 and AfG2), AfG2 is found in greater quantities, but is less toxic, while AfB_1 is the most toxic, referred to as class1 carcinogen (WHO, 2005; Thrasher, 2012). Aflatoxins display potency of toxicity, carcinogenicity, mutagenicity in the order of AfB1>AfG1>AfB2>AfG2 (Bennett *et al*., 2003; Cortes, 2010). The toxicity of aflatoxin B1 varies in different animals from extremely susceptible among sheep, dogs and rats to resistant species found among monkeys, chicken and mouse as reported by Bbosa *et al*. (2013). Aflatoxin B1 are mainly metabolized in the liver to a reactive epoxide intermediate or hydroxylated to M_1 form in animal tissue and/ or milk (Wild &Montesano, 2009; Wu & Khlangwiset, 2010).

Aflatoxin B1 is the most prevalent type present in contaminated feeds, forming 60-80% of the total aflatoxin content, while aflatoxins B2, G1 and G2 are generally not reported in the absence of aflatoxin B1. The reason being *Aspergillus flavus* and *Aspergillus parasiticus,* which are the main producers of aflatoxin B1 and B2 predominate due to their wide favourable environmental conditions and utilization of a wide variety of substrates (Boutrif, 1998). Aflatoxins B2 and G2 are typically present in much lower quantities (European commission, 2016).

2.5.6 Implications of aflatoxin consumption in the human food chain

Long-term exposure of animals to aflatoxins has several health effects that are manifested as potential carcinogens which may cause harm to body organs, particularly the liver and kidneys resulting into cancer and AFB1 is particularly carcinogenic to humanity being; the ability of aflatoxin to cause liver cancer is significantly enhanced in the presence of infection with hepatitis B virus (WHO, 2000). Aflatoxin is mutagenic on bacteria, genotoxic, and has the capacity to bring about birth defects in children; stunted growth-with the contribution of other factors to cause growth faltering; examples include persistent diarrhoea, infectious diseases, malnutrition; aflatoxins are the main causes of immunosuppression among infants, resulting into decreased resistance to infectious diseases (WHO, 2000).

High dose consumption of aflatoxin end up in acute poisoning that is life threatening to rabbits, through its effects on the liver. Acute liver failure that is observed through such

signs as jaundice, nausea, lethargy and probably death, have been reported in human populations from the 1960s (Mutegi *et al*., 2018). The most recent reported cases of death due to aflatoxins occurred in 2016 at Tanzania. Intake of food containing concentrations of 1 mg/kg or higher has been reported to cause high aflatoxicosis rate. Based on past outbreaks it has been estimated that consumption for over a period of 1–3 weeks, an AFB1 dose of 20–120 ppb per day is acutely toxic and potentially lethal (Mutegi *et al*., 2018).

Acute episodes of disease show specific signs that include haemorrhage, oedema, alteration in digestion, absorption and/or metabolism of nutrients, and may end up in death (Sharma, 2011, Gupta, 2011). Acute dietary exposure to aflatoxin has been reported in epidemics due to hepatic injury (Mutegi *et al*., 2018). Evidence of the occurrence of aflatoxicosis in humans has been reported to have occurred worldwide particularly in the third world countries that include Taiwan, Uganda, India and Kenya (Mutegi *et al*., 2018).

Aflatoxin has been implicated to interfere with nutrition in a relationship manifested in intake of aflatoxin and rate of growth in children as it causes nutrient modification particularly in vitamin A or D in most animals, making these nutrients not to be available for the body physiological functions, leading to nutrient deficiencies (Thrasher, 2012).

In rabbits, acute aflatoxin poisoning show signs such as anorexia, depression, lethargy, reduced feed intake and feed conversion efficiency, high drop in milk production, gastrointestinal disorder such as ascitis, icterus, tenesmus, abdominal pain, bloody diarrhoea, weight loss, jaundice, abortion, hepatoencephalopathy, blindness, walking in

circles, ear twitching, frothy mouth, photosensitization, bleeding and death (WHO, 2018; Thrasher, 2012;). Beside lack of appetite, weight loss, decreased egg production, leg and bone problems, poor pigmentation, fatty liver, kidney dysfunction, bruising and death, suppression to natural immunity and susceptibility to parasitic, bacterial and viral infections can occur (FAO, 2017).

Aflatoxins and its numerous types, metabolites and their products that include the reactive oxygen species like the AFB1-8, 9-epoxides have the capacity to interfere with the optimum functioning of nerve cells through formation of oxidative stress factors, DNA adducts, protein adducts, mitochondrial directed apoptosis of the nerve cells as well as inhibiting the synthesis of protein, RNA and DNA (Thrasher & Grawley, 2012). Aflatoxins are at the same time known to cause damage to mitochondrial DNA structure and function, which results into defective oxidative phosphorylation in the brain cells (Thrasher & Grawley, 2012; Halliwell 2007).

Aflatoxins are produced in cereal grains and forages before, during and after harvest under various environmental conditions. As a result of the diversity of their toxic effects and synergetic properties, they are a health risk to consumers of contaminated feeds (Yiannikouris & Jonany, 2002). Mycotoxins are a problem the World over (Lawlor & Lynch, 2005) and are estimated to contaminate 25% of the World's food crops, although, there are great variations among environmental variables, monitoring and control technologies of nations.

Aflatoxin can cause physiological disorders and histological changes in animals that can result in reduced production of milk or meat (Boonyaratpatu *et al*., 2002). Aflatoxins exert their effects on rabbits through alterations in nutrient content by way of reactive attachments- especially to unsaturated nutrients, nutrient absorption when the mucosa of digestive tract is corroded and metabolic changes through alteration of endocrine and neuroendocrine functions (Waldner & Lalman, 2010).

A number of studies on rats and rainbow trout have shown that aflatoxin, especially AfB1, is the leading cause of cancer (IARC, 1993). In 2004, acute aflatoxicosis killed 125 people in Machakos and Kitui Districts (now counties) of Eastern Kenya, who consumed aflatoxin, contaminated maize (Probst *et al.,* 2007).

2.5.7 Aflatoxin effects on Nucleic acid

Aflatoxins consumption by animals may lead to damage to protein formation due to disruption of nucleic acid or the process of protein synthesis (Kensler, *et al*., 2011). After activation to reactive Aflatoxin B1- 8, 9-epoxide form, it binds to biological molecules that include essential enzymes, blocks RNA polymerase and ribosomal translocase resulting into inhibition of protein synthesis (Omar, 2013)

Aflatoxin B1 is metabolically activated by cytochrome P450 enzymes in the liver to its reactive metabolite, AFB1- 8,9-epoxide, which binds to macromolecules resulting in formation of adducts such as DNA adduct, which may lead to mutations and carcinogenesis. The AFB1-8,9-epoxide can further be converted to AFB1-8,9-diol that

specifically binds to lysine in albumin and form AFB1-lysine adducts, which has been validated as a biomarker of human exposure to aflatoxins (Qian *et al*., 2013)

The aflatoxins and its metabolites especially the AFB1- 8, 9-epoxide affects the telomere length during the cell division and the various check points in the cell cycle thus affecting the signaling pathways and the regulatory processes of the cell cycle. The extent of aflatoxin binding to DNA and its damage, affects the level of different protein synthesis in the cell cycle and the apoptotic pathways such as c-Myc, p53, pRb, Ras, protein kinase A, protein kinase C, Bcl-2, NF-kB, CDK, cyclins and CKI that contribute to the life or death decision making process of the cell (apoptosis). They may also contribute to the deregulation of the cell proliferation leading to carcinogenesis. The reactive aflatoxin- 8, 9-epoxide can affect the mitotic (M) phase, growth process and DNA synthesis in the cell cycle leading to carcinogenesis (Bbosa *et al.,* 2013). Metabolization of aflatoxin by CYP3A4 and CYP1A2 enzymes create the highly reactive ORS, aflatoxin -8, 9 exposide, which binds to DNA and albumin in serum that induces carninogenesis (Wu & Khlangawiset, 2010).

2.5.8 Effects of aflatoxin on Rabbit fertility

Fertility is the ability of an animal to conceive and maintain pregnancy at the appropriate time in relation to ovulation (Santos *et al*., 2010). During the recent years, the studies on harmful effects of aflatoxin on animals excluded those of direct interference of reproduction but included indirect ones in relation to physiological systems and their

processes. But, more current animal studies suggested inclusion of aflatoxins' direct effects on reproductive toxicity among both male and female rabbits particularly their damaging effects on gametes and physiology. Investigation of aflatoxin exposure *in utero* and the monitoring of foetal development parameters manifested their retarded growth, decreased foetal or egg weights and reduced foetal length among various rabbit species (Peles *et al*., 2019). Aflatoxicosis in piglets has been reported to cause growth retardation, impaired peripheral immune efficiency and thymic involution, leading to early mortality (Mocchegiani *et al*.,1998), while exposed broiler chicken brought about embryonic death and diminished immunity (Rawal *et al*., 2010). Aflatoxin has spermatotoxic effects, whereby it impacts negatively on the physiology and morphology of the spermatozoa. Aflatoxin interferes with the rabbit male reproduction by changing spermatogenesis, epididymal and Leydig cell functions, as well as the reduction of the level of secretion of testosterone and general fertility, similar to what has been reported among other animals including rats, fowls and cows (Agnes & Akbarsha, 2003).

Among the female rabbits, aflatoxin diminishes fertility of oocytes by disrupting their maturation by way of epigenetic modification and cause oxidative stress, high autophagy and apoptosis (Liu *et al*., 2015). In poultry, aflatoxin reduces egg yield and quality, affects liver metabolism and function and lesions in layer DNA, inhibit protein synthesis, lipogenesis and cause reduced feed intake and digestibility (Jia *et al*., 2016).

2.6 Aflatoxin Control and Management

Kenya bureau of standards (KBS) is the government body mandated to control food and feed quality and safety. Its maximum allowable aflatoxin level in food was 20 ppb (KBS, 1988), similar to that of FDA. But after the aflatoxin outbreak of 2004 in Eastern Kenya, the allowable level was lowered to 10 ppb (Gitau, 2004), making it more difficult to achieve, because it will almost ensuring that much of the grain is rejected. Home grown food and compounded feeds by subsistence farmers and those sold to vendors, small scale industries and processors are not monitored neither are these handlers aware of aflatoxin risks nor do they have a laboratory to analyze. Large scale food processors, which mill cereals for both human food and livestock feeds, however, are aware of aflatoxin risks and have developed testing laboratories for screening their raw materials (Kangethe, 2011).

Due to Kenya Bureau of standard's lack of capacity in terms of labour and facilities to monitor and reinforce feed quality in the country, the Government, through extension service, tried to educate farmers on the importance of proper drying and handling of cereals, particularly maize, but this knowledge has not translated into the reduction of aflatoxin, particularly in the prone areas (Daniel *et al*., 2011). This has made consumption of aflatoxin contaminated feed from grain and grain by – products by both livestock and human beings in Kenya and Africa a health risk.

The Kenya Government, through KALRO, in collaboration with IITA has developed an aflatoxin bio-control compound called Alfasafe KEO1 in 2017. This product will be

produced in Kenya and the farmers will apply the chemical on the maize at the farm to control the fungus which normally produces aflatoxin. It is hoped that the product will reach to the farmers so as to probably reduce the production of aflatoxin at farm level.

The African countries, have established and mandated the partnership for Aflatoxin Control in Africa (PACA), based in Ethiopia, to develop co-regulatory procedures for aflatoxin in maize for Africa (http://www.aflatoxinpartnership.org). This work is yet to be completed.

Grain is either harvested when it is already contaminated with aflatoxin or the contamination occurs due to poor handling and storage conditions, particularly high moisture content and frequent climatic changes from dry to moist warm conditions favourable for the growth of the toxin.

2.7 The Liver and its role in steroid hormone synthesis

The liver is a non-endocrine organ in an animal's body that carries out vital roles in the regulation of hormone synthesis, balance and other functions in an animal's body. The liver plays a central coordination role in metabolism for the production of important substrates for secretion of hormones that influences reproduction in animals. Among these hormones, Insulin growth factor is important for the regulation of cell growth and development of the stimulation of growth hormone, while oestrogen receptors in the liver are critical for maintaining fertility (Torre *et al.,* 2011). Mice lacking oestrogen receptors have been

found to be low in plasma insulin growth factor-1, which is necessary for the growth of the lining of the uterus and influences the animal's oestrus cycle (Torre *et al*., 2011).

The liver plays a vital role by producing about 80% of cholesterol, a substance that is necessary for the synthesis of steroid hormones, produced in the mitochondria and transported for storage at the endoplasmic reticulum of the hepatic cells, and is essential for mammalian cell function, integrity and structural cell components where it ensures cell permeability and fluidity of the membrane (Komati *et al*., 2017). The liver plays an important role by maintaining cholesterol homeostasis through regulation of absorption and synthesis (Komati *et al.*, 2017).

The primary source of cholesterol is the diet consumed by the animal, then absorbed via the intestinal mucosa and taken to the liver, which distributes it to other organs. Cholesterol is formed from high density lipoproteins whose receptors mediates the high density lipoproteins-cholesterol ester uptake (Sedes *et al*., 2018). Cholesterol synthesis starts with acetyl-CoA that is transported from the mitochondria to the cytosol. Several reactions yield Hydroxy-methylglutaryl-CoA (HMG-CoA), which will be converted into mevalonate by HMG-CoA reductase and several other steps to result into cholesterol (Sēdes *et al*., 2018).

2.7.1 Steroidogenesis

The substrate for steroidogenesis, cholesterol, is transported into the mitochondria by steroidogenic acute regulatory proteins (Supriya *et al*., 2014). Cholesterol, having been

synthesized in the mitochondria from lipoproteins obtained from plasma, is converted into a basic ring named cyclopentanoperhydrophanthrene, depicted by pregnenolone (C-21 steroid), a basic compound for the biosynthesis of steroid hormones by the aid of a number of enzymes, chief of which is aldosterone synthetase (Supriya *et al*., 2014). A sketch of this process is given in figure 2.4 below;

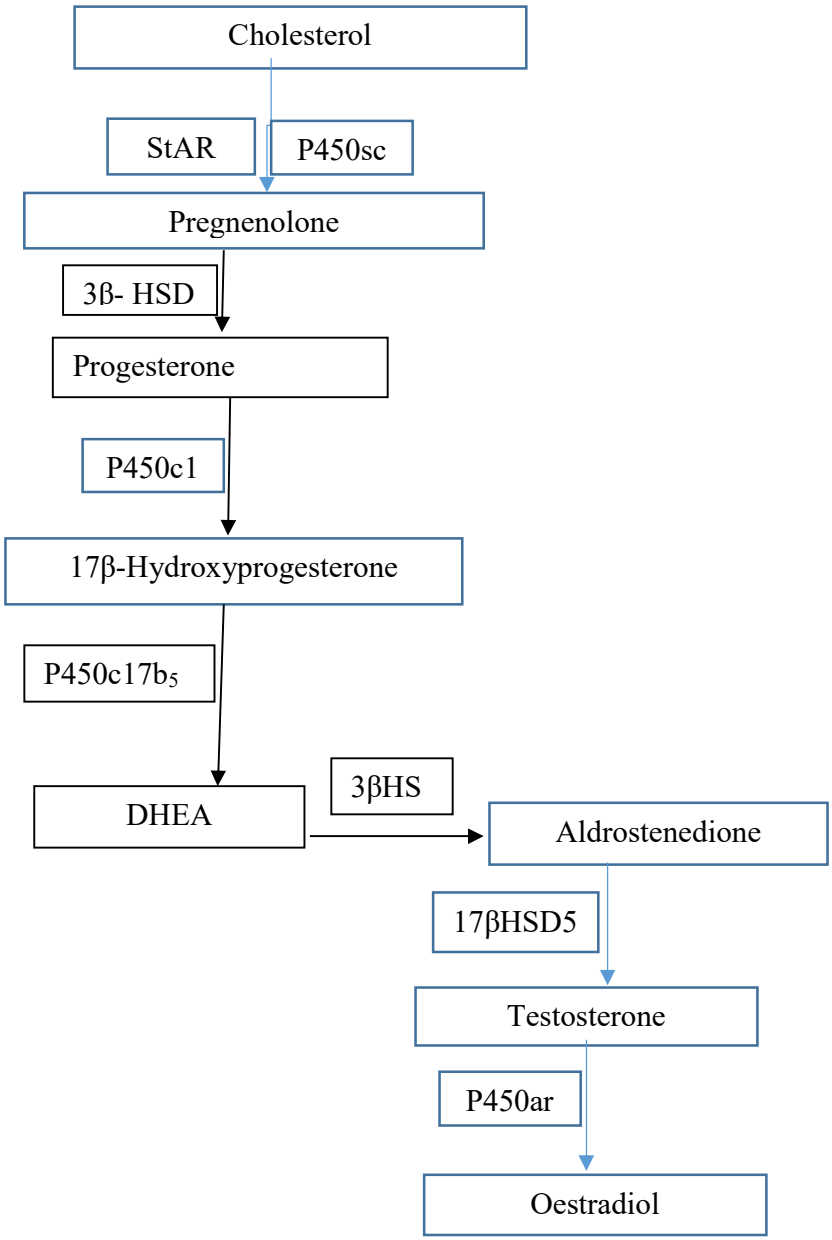


Figure 2.4: The female steroid hormone biosynthesis pathway (Acconcia & Marino, 2017)

Aflatoxins are produced in cereal grains and forages before, during and after harvest under various environmental conditions. As a result of the diversity of their toxic effects and synergetic properties, they are a health risk to consumers of contaminated feeds (Yiannikouris & Jonany, 2002). Mycotoxins are a problem the World over (Lawlor & Lynch, 2005) and are estimated to contaminate 25% of the World's food crops, although, there are great variations among environmental variables, monitoring and control technologies of nations.

Aflatoxins exert their effects on rabbits through alterations in nutrient content by way of reactive attachments- especially to unsaturated nutrients, nutrient absorption when the mucosa of digestive tract is corroded and metabolic changes through alteration of endocrine and neuroendocrine functions (Waldner & Lalman, 2010). The current work, investigated aflatoxin militating effects on physiological order and histology among rabbits that can result in reduced kitten parturition and the overall rabbit meat production (Boonyaratpatu *et al*., 2002).

This investigation arose based on earlier work on commercial concentrates in urban Centres in Kenya which revealed that most commercial feeds contained up to 550ppb of aflatoxin (Kangethe & Lang'a, 2009). Feed samples taken by this author, showed that 81% of the feed were positive for aflatoxin, with Nakuru and Nairobi urban centres showing an average of 280ppb of aflatoxin B1 alone. It therefore raised queries regarding the effect of aflatoxin on the reproductive system of rabbits.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Research study permission

Ethics clearance for the current research was obtained under the code No UEAB/12/8/2018 of August 2018.

3.2 Study Site

The current study was carried out at the University of Eldoret, within the coordinates 0°31'N (Latitude), 35°16'E (Longitude) and at an altitude of 2100 meters above sea level. The location has an average annual temperature of 17°C and a bimodal annual rainfall of 1100mm. The study was carried out in a house constructed to ensure a room temperature range of 18 - 22^{o}C, which was monitored using a thermometer, and a relative humidity of between 53 - 60% (checked using hygrometer) as recommended for rabbits by Asemota & Orheruota (2020). The house was designed and constructed to facilitate 12:12 hour to allow the cyclicity of light and darkness that matches the physiological system of the reproductive axis which has been reported to play a crucial role in maximizing reproduction. The rabbit house allowed maximum ventilation and air movement to enable the rabbits to thrive well, but without windy condition.

3.3 Experimental rabbits

The current study used rabbits because they have been reported to be useful animal in the extrapolation of human physiological conditions, while their phenotypic gene expressions and importance of histopathological and immuno-histochemical expressions in their organs may point to similar effects to those of human being (Shiomi, 2009). However, the current

study utilized rabbits to determine the effect of aflatoxin on rabbit reproductive system but serve well as a physiological model for other species of animals.

A total of sixteen New Zealand white breed of female rabbits (*Oryctolagus cuniculis*) aged three months were used for the study (Appendix 3). The rabbits were acquired from a single source, Tatton farm of Egerton University demonstration and research unit in Nakuru County, which kept the pure breed under confinement and fed on commercial pellets/or mash. The farm produces rabbits for research, teaching and supply to both farmers and other institutions for meat production, breeding or research. The rabbits had been familiarized to cage rearing and consumption of concentrate mash and/or pelleted diets. Upon arrival to the study site, the rabbits were each assigned a number that was written on a plastic ear tag and tagged on to the ear using an ear tag applicator and the ear was notched too for the same number. The number on the tag was written using a permanent marker and it was not removed nor erased until the end of the study.

3.4 Housing and care for experimental rabbits

The experimental rabbits were kept inside the house which had wide windows closed with a combination of welded and chicken wire mesh to ensure free air circulation, but protected from entry of birds and predators. The room was reinforced against rodents, though rodenticides were used to check any possible presence of rats. The rabbit house was well ventilated, with sufficient light through translucent iron sheets at the roof and wide windows to ensure 12:12 hour light: dark cycle, with a room temperature of 18 – 22^{o}C. The

rabbit house was thoroughly cleaned, disinfected and anti-mite dust applied to kill any possible mites in the rabbit house. Saw dust was then spread on the floor, under the rabbit cages, before a second disinfection. The saw dust was meant to absorb moisture and assist in the maintenance of both the relative humidity and temperature at 20 – 30% and room temperature levels respectively to minimize offensive odour that would easily create discomfort in the rabbit house. High relative humidity makes air to be murky and uncomfortable, reduces evaporative surface and perspiration heat loss making the animal to be uncomfortable. The saw dust was removed every week to ensure the least rotting which could have easily caused some infection on the rabbits. Retaining the saw dust for too long would likewise be a source of increased relative humidity and eventually temperature in the rabbit house and could have easily increased their discomfort.

Rabbits for each treatment were kept in cages built to measure 80 X 50 X 30 cm as designed and recommended by the department of livestock and fisheries (GoK, 2009). These rabbit cages were built using timber frames whose sizes measured 3”x 2’’, with welded wire mesh on the sides and the floor. The wielded wire mash at both the sides and floor of the cages were reinforced with chicken wire so that the rabbit feet didn’t pass through, but also walk on the floor base without injuries on their feet.

3.5 Experimental design

The study ran for 45 days during which time experimental data was collected. The study used experimental laboratory based design for data collection. A total of sixteen rabbits of

three months of age were randomly distributed to four treatments using completely randomized design procedure. The designed experiment had 4(replicates) rabbits for each treatment in a repeated measurement study. The rabbits were allowed seven days to adapt to the experimental conditions that included new diets, regular weighing and ear tag checking and the social order in the cages. The baseline data including blood samples for hormone determination, was collected on the seventh day of acclimatization of the rabbits. The mean rabbit weights for each treatment were; 3.08, 3.13, 3.12 and 3.13 kg for treatments 1, 2, 3, and 4 respectively. The study treatments involved the diet without aflatoxin while treatments 2, 3 and 4 had total aflatoxin incorporated at different levels as follows; treatment1–control (concentrate mash diet without aflatoxin), treatment 2–concentrate mash containing 100 ppb of aflatoxin, treatment 3- concentrate mash containing 200 ppb of aflatoxin and treatment 4- concentrate mash containing 400 ppb of aflatoxin in feed dry matter.

The independent variables of interest were the levels of total aflatoxin (aflB1,aflB2, aflG1 and aflG2) in the concentrate diets consumed by the rabbits, while dependent variables included the hormone levels in the blood plasma of rabbits and the histological changes caused by aflatoxin on the hypothalamus, pituitary gland, ovaries, liver, the uterus and fallopian tube.

3.6 Sample size

The study used a homogeneous set of New Zealand white breed of Rabbits for the study. The sample size required to test the hypothesis demanded the selection of a suitable

formula because the effect of breed-to-breed variation on the measurement was avoided by using a single breed. It was therefore important to obtain an optimum sample size necessary for the interpretation of the data obtained without bias. The study used the sample size calculation developed for clinical studies as proposed by Fleiss (2007). This targets the achievement of 60% (β=0.6) accuracy at a critical limit of 95% (α=0.05) for statistical significance. The formula used for the calculation was as given below;

$$n = C\frac{(p_c q_c + p_e q_e)}{d^2} + \frac{2}{d} + 2$$

$$n = 7.85\frac{(0.4 \times 0.3) + 0.4 \times 0.6)}{0.6^2} + \frac{2}{0.6} + 2 = 14$$

Where; $P_c = q_c - 1$, $q_e = 1 - p_e$, d=difference between pc and pe expressed positively

Pc=the estimates for proportion for the control group exhibiting the occurrence

Pe = the desired proportion of the experimental groups exhibiting the activity

C is a constant, expressed in this case at 7.85 for α=0.05 and β=0.8

3.7 **Feeding of the Rabbits**

The persons attending to the experimental rabbits always wore protective clothing that included laboratory coats, gumboots and aspirators at any time when working to minimize any possible entry of aflatoxin into their bodies, which has been reported to enter through

wounds or nostrils by inhalation. Each treatment group of rabbits were given 400 gm of feed twice daily; at 8.00 am and 3.00 pm using stable broad based earthen pots, as feed troughs, to ensure they didn't topple over incase the rabbits stepped on them. The amount of concentrates fed was based on the average live weight of the rabbits. The amount of feed provided was arrived at depending on the daily requirements based on the live weights of the rabbit and a 7-13 hour interval to enable the rabbits consume sufficient amount of feed during the day. Feeds were weighed using an electronic balance (Model TSC-A4, made in China) before it was provided in the feed trough. The twice daily feeding was to ensure that the troughs were not filled to the brim for purposes of minimizing spillage and to avail feed to the rabbits every time of the day, as well as to ensure a stable continuous and optimum feed digestion for the intake of nutrients and aflatoxin to meet the requirements of the study. Underneath the feed troughs were gunny bags for collecting feed spillage. The feed that remained and those from spillage were weighed every morning and feed intake for the previous day determined by difference. The rabbits were supplied with clean tap water *ad libitum* in earthen pot bowls. The pots were emptied and refiled with fresh water regularly to ensure it remained clean.

3.8 Preparation of experimental diets

3.8.1 Growing of *Aspergilus flavus*

Rabbit diets were compounded by Chania feeds factory based at Thika, Kiambu County in Kenya, with a proper balance of nutrients for growing rabbits, but free from aflatoxin

binders. The feeds were delivered to Everest store and laboratory technologies located in Kabete-Nairobi, who incorporated the total species of aflatoxin (aflB1, aflB2, aflG1 and aflG2) into the diets in the quantities required for each experimental treatment as follows; incorporation of aflatoxin in rabbit feeds in the quantities required began when 10 petri dishes of pure culture of *Aspergillus flavus* spores was grown under controlled environment for five days. These pure culture in petri dishes were used to dominate the environment where reduced 30 kg of maize and 10 kg of peanut grain particles were spread on trays. The crushed maize and peanut grains of high quality, which were water stressed, were spread on trays measuring 3 x 2 x 4ft. The grain particles on plastic trays were kept on racks in a chamber under the following environmental conditions; temperature regime of 30 – 33^{o}C, relative humidity of 70 – 80%, pH range of 6.0 – 7.0 and water activity of 0.6 – 0.8 (amount of water available for microbial action). Racks were used to spread out the crushed grains to increase the surface area for optimum contact and growth of *Aspergillus flavus* on the grains.

The open trays of grain with *Aspergillus flavus* were placed in open chambers, the heat was controlled and a plate of water placed inside every four days to maintain the relative humidity. The highly contaminated crushed maize grain was then ground, tested for aflatoxin using ELISA assay and thereafter stored at 40^{o}C in air tight containers.

3.8.2 Incorporation of *Aspergilus flavus* in experimental diets

The total rabbit feed mash required for the current study which was 120 Kg, were acquired on order from Channia feed factory at Thika and was ascertained that it didn't have aflatoxin or had at least the minimum of less than 5ppb and were free from aflatoxin binders. The first 30 Kg of dry feed for the control treatment was weighed out, kept in a bag and taken away from the laboratory to avoid any exposure to aflatoxin, but stored under dry conditions in store awaiting transportation to the experimental site.

The aflatoxin contaminated crushed maize grain material was mixed with vegetable oil in readiness to be spread on the rabbit feeds in the controlled chamber. The oil was meant to facilitate the sticking of *Aspergilus flavus* on the grain and it also serves as its substrate. The 90 Kg of feeds, remaining after the removal of that for the control, were spread out in the controlled chamber under the environmental conditions given earlier. Then all the crushed contaminated grain was spread over the feeds, mixed thoroughly and environmental conditions monitored. Feed sampling and aflatoxin testing was carried out every four days by quantitative ELISA range of 1.75 – 141.75 ppb with 3 dilutions in each assay. The moment aflatoxin level of 100 ppb was attained, the second batch of feeds of 30 Kg were weighed out, dried to 14^0C, packaged and stored in air tight store under room temperature.

The remaining 60 Kg of the feeds were returned to the environmentally controlled chamber and the *Aspergillus flavus* allowed to grow. The ELISA test for aflatoxin content was carried out every 4 days until it attained the 200 ppb. From this, another 30 Kg of the feeds

was removed dried to 14^{o}C and kept in an air tight bag and kept under dry room temperature conditions. The process was again repeated to grow aflatoxin level to 400 ppb. The exercise of incorporating aflatoxin into the treatment diets and several ELISA tests were labour intensive, time consuming and demanded patience to ascertain that the right aflatoxin content was incorporated into the feeds. Some time it demanded the separate growth of more *Aspergillus flavus* to be added into the diet to quicken the process. The feeds were dried quickly and thoroughly to attain 14^{0}C immediately the aflatoxin content required for the particular batch of feeds were attained.

3.9 Precautions taken on handling of feeds during data collection

During the preparation of aflatoxin laced diets and feeding of rabbits, precaution was taken to avoid accidental exposure to aflatoxin. The housing area of the experiment was restricted to unauthorized human access to minimize possible rabbit disturbance during the trial and to reduce the risk of inhalation of aflatoxin. The feed compounder, animal attendant and researcher were dressed in protective clothing that included gumboots, gloves, laboratory coats, aspirators and goggles at any time at work in the animal house. The rabbit housing unit was located in isolation, away from other animal species and grain stores. The rabbit feeders were cleaned daily to ensure that they were free from other contaminants. The feeds were labeled correctly and kept at specific points away from rabbit cages and other possible contaminants in order to minimize any possibility of a mix up during feeding by the animal attendant or any other person throughout the study period.

3.10 Ovulation synchronization of rabbits

On the seventh day of the acclimatization period, all the rabbits were synchronized for ovulation through intramuscular injection of 1 ml of fertirelin, a gonadotropin releasing hormone agonist. The site of injection which was the shoulder muscle was sterilized with alcohol, injected using a 21 gauge needle and massaged with alcohol socked piece of cloth. The hormone was administered on the seventh day when the rabbits had acclimatized to the study environment, as there is evidence that this influences hormone secretion(Bronson & Heideman 1994). Gonadotropin releasing hormone agonist (substance that acts like GnRH) is a synthetic model of the naturally produced decapeptide releasing hormone from the hypothalamus, but specific alterations that are double and single substitution of the amino acid at carbon position 6, alkylation of amino acid at carbon number 9 and deletion of the one in carbon number 10 have taken place (Urner, 2018; Lehne & Rosenthal, 2014). The GnRH agonist was obtained from a Menengai veterinary supplier in Nakuru. The chemical structure of GnRH has the following components;

$C_{60}H_{73}N_{15}O_{13}$

Gonadotropin releasing hormone agonist stimulates the pituitary gland to secrete FSH and LH that are with the growth and function of the reproductive organs in female animals (Lehne & Rosenthal, 2014). The aim of the substitutions and deletions were to increase the shelf life of the agonist (Gardner & Symon, 2017).

Oestrus synchronization in rabbits has been shown to alter physiological functions as reported through increase production by way of reduced kiddling interval and general prolificacy in a population (Rebollar *et al*., 2006). These was necessary for the current study to ensure that all the female rabbits were at the same oestrus status at the beginning of the study and observed as the reddening of the vulva and the discharge of thin clear consistent mucus on the 7 – 8 th day after synchronization. Oestrus synchronization in rabbits was necessary to ensure that the results did not vary due to the rabbits being at different stages of oestrus cycles.This ensured that the rabbits were physiologically at oestrus phase at the beginning of the study.

3.11 Bleeding of the rabbits

The blood samples for hormone assay were obtained from the central ear vein of the rabbits in each treatment after every 4 days. The ear was punctured using a 21 gauge needle and the blood collected into 5 ml vacutainer tubes. The number of each rabbit and date of sample collection were written on the tubes, packaged in a carton before being transferred to the Nairobi Annex laboratory in Eldoret town immediately for further processing.

Upon receipt in the laboratory, the blood samples were centrifuged (using Hitachi general purpose, high performance-CP100WX model) at 3000 revolutions per minute for 3 minutes to separate the serum. The serum was decanted into cryovials and appropriately labeled before being stored in the refrigerator at 4 -8^{o}C where it can be kept for up to 6 Months before analysis.The current samples were kept for two months before it was analyzed.

Once it was time to analyze the samples, they were first removed from the freezer and thawed to attain room temperature of about 22^{o}C at which point the tests were carried out.

3.12 Hormonal assay

Blood samples were collected before the beginning from the rabbits in each treatment before the beginning of data collection for analysis to obtain the baseline information on the oestradiol, FSH and LH hormone quantities. The same information was obtained from the blood that was drawn from the rabbits on the 2nd, 3rd, 4th and 7th week of data collection in the experiment.

The blood sample was taken using closed bleeding system recommended by WHO (2010). During the bleeding, the rabbit was picked gently from the treatment cages one at a time by a handler and held up by the back of the neck using one hand, while the other hand supports at the ram, then a phlebotomist drew the blood (Appendix 5). The area where the needle was to be inserted was disinfected using 70% isopropyl alcohol solution. The blood samples were drawn by puncture of the ear's medial ear vein of the rabbits. The puncture was done on the left side of the ear using a 21 gauge needle and the blood drawn into 5ml disposable syringes. The blood samples from each rabbit were transferred into a heparinized vacutainer that was labeled and the number of the rabbit was recorded. The vacutainers were packed in a carton and taken to Nairobi Annex laboratory at Eldoret for the determination of hormonal levels. The blood samples were processed for hormone determination immediately upon arrival into the laboratory.

3.12.1 Oestradiol immuno-assay

The oestradiol hormone immunoassay in the current study was carried out using Eclecsys Estradiol III immunoassay analyzer. The procedure used electrochemilumiscence immuno-assay. The immunoassay of oestradiol is used clinically to observe functional indicators in the hypothalamus – pituitary gland - gonadal axis. The Eclecsys Estradiol III immunoassay analyzer employs a competitive test principle that uses two monoclonal antibodies directed at 17β-oestradiol, which competes with the added oestradiol derivative for the binding site on the biotinylated antibody.

Upon delivery to the Nairobi hospital Annex laboratory at Eldoret, the 5 ml blood sample was allowed to settle for 30 minutes to facilitate proper separation. The blood sample was centrifuged, using Hettich EBA III model (United Kingdom) at 3000 rpm for 15 minutes to facilitate plasma collection. After settling, the supernatant was separated and a sample amount of 25µl was then transferred using Pasteur pipettes to a test tube and incubated with 9 mL oestradiol specific biotinylated antibodies. Secondly, 6.5 mL of streptavidin coated micro particles and oestradiol derivatives were added to have the vacant sites of biotinylated antibodies become occupied, with the formation of an antibody-hapten complex, which interacted with biotin and streptovidin. The reaction mixture was transferred into measuring cells where the complex was captured magnetically on the surface of the electrodes. The magnetically captured complex induced chemiluminescent emission, which was then measured by photomultiplier. The resulting outcome were

determined by the analyzer automatically by calculating the oestradiol concentration in the sample and displayed the results on the liquid crystal display screen.

3.12.2 Follicle Stimulating Hormone immuno-assay

Upon delivery to the laboratory, the 5 ml blood sample was centrifuged, using Hitachi EBA III model (United Kingdom) at 3000 rpm for 15 minutes to facilitate plasma collection. It was allowed to settle for 30 minutes to facilitate proper separation of plasma then; 150µl of supernatant was removed and transferred using Pasteur pipettes to a test tube containing the detection buffer. The testing in the current study used Ichroma™ FSH, a fluorescence immuno-assay facility that measures follicle stimulating hormone concentration in plasma. It uses antigen-antibody interaction and fluorescence technology. The sample and detection buffer were mixed and loaded onto a sample well on the cartridge, this formed a complex of anti-FSH-antigen on the membrane on the cartridge. The more the FSH in plasma, the more the complexes that accumulate on the membrane. The Ichroma™ reader scanned the intensity of the fluorescence on the membrane, and then displayed the FSH concentration on the liquid crystal display (LCD) screen of the reader. The lead of the detection buffer tube was closed and the mixture shaken 10 times. Using a Pipette, 75 µl of the mixture was taken and dispensed into the sample well on the test cartridge and left to settle at room temperature for 15 minutes. The sample was then inserted into the cartridge holder from where it was loaded onto the test cartridge in readiness for the Ichroma™ reader, from Boditech Med (London, United Kingdom). The select button on the reader was pressed on

to start the scanning process. The Ichroma™ **r**eader calculates the test results automatically and displays the hormone concentrations of the sample in terms of mlu/ ml. The test results were then read on the display screen of the reader, which showed the amount of follicle stimulating hormone by fluorescence immuno-assay (Bioditech med-Inc. 2014) system which uses antigen-antibody interaction and fluorescence technology.

3.12.3 Luteinizing Hormone immuno-assay

The testing equipment, Ichroma™ LH, is a fluorescence immunoassay cachet for the quantitative determination of luteinizing hormone levels in the screening or monitoring of fertility issues and the functioning of the reproductive organs. Luteinizing hormone determination is an immunoassay test that uses fluorescence technology, such that, the antibody in the detection buffer binds to the luteinizing hormone in the blood sample. The antibody-antigen complex are immobilized and captured on the test strip. The more the LH antigen in the blood, the more the antigen-antibody complexes accumulated on the test strip and this will signal the intensity of the fluorescence as an indication of the quantity of luteinizing hormone that will be shown on the LCD screen.

Once the 5 ml blood sample was delivered to the laboratory, it was centrifuged, using Hitachi EBA III model (United Kingdom) at 3000 rpm for 15 minutes to facilitate plasma collection. The sample was then allowed to settle for 30 minutes to facilitate proper separation of plasma. Then, 150µl of supernatant was taken from this sample and transferred using Pasteur pipettes to a test tube containing the detection buffer. The lead of

the detection buffer tube was inserted and the mixture shaken 10 times. A sub-sample of 75 µl of the mixture was taken and dispensed into the sample well on the test cartridge using a Pipette and left to settle at room temperature for 15 minutes. The sub-sample was then inserted into the cartridge holder from where it was loaded onto the test cartridge whose results were to be displayed from the Ichroma^{TM} reader (Boditech Med. London, United Kingdom). The select button on the reader was pressed on to start the scanning process. The Ichroma^{TM} Reader calculates the test results automatically and displays the luteinizing hormone concentrations of the sample in terms of mlu/ ml. The test results were then read on the LCD display screen of the reader, which showed the amount of luteinizing hormone by fluorescence immunoassay (Bioditech med-Inc., 2014) system which uses antigen-antibody interaction and fluorescence technology.

3.13 Harvesting and processing of rabbit organs

At the end of the animal treatment period, one rabbit was picked at random from each of the four treatments to be sacrificed for the examination of the hypothalamus, anterior pituitary gland, ovary, liver, uterus and fallopian tube for histology. A total of four rabbits were taken to Moi teaching and referral hospital's anatomy and physiology laboratory where microscopic tissue examination for aflatoxin effect was carried out. The rabbits were kept in air tight glass cages for 30 minutes with a piece of cotton wool inside that was socked with ether to bring them into unconscious (over dose) state to facilitate humane sacrificing. The rabbits were humanely sacrificed by hitting the head using a blunt object

after they became unconscious, and the hypothalamus, pituitary gland, ovary, liver and uterus were harvested. The hypothalamus and pituitary gland were accessed by first gently removing the skin from the head immediately the rabbits were sacrificed. The skull was gently broken through using a hack saw. A solution, with a concentration of 10% buffered formalin (Appendix 6) was carefully infused through the opening into the skull tissues and the whole head preserved for a period of one week to allow time for stabilization. Buffering of formalin facilitated its wide mobility and permeability into rabbit cells(Sheehan & Hrapchak 1980). Thereafter, the skull was broken through gently to take the whole brain organ from which the hypothalamus and the anterior pituitary gland were extracted for the preparation of histological sections.

The abdomen was opened and the ovary, uterus and fallopian tube were harvested for preparation of histological sections in readiness for microscopic examination. Tissue preparation for histological work was carried out in MTRH's anatomy and physiology laboratory according to procedures given by Abert *et al*. (2015) as stated below;

3.13.1 Fixation of cells and tissues

In the current study, the fixative solution containing 10% neutral buffered formalin was used to fix the tissues. This was important to preserve the cells permanently within tissues as in original state immediately following the harvest. Fixation is a chemical process through which biological tissues are preserved from decay arising possibly from autolysis or putrefaction, and fixation also offers mechanical strength or stability to tissues. The

tissues were treated with the fixative at the ratio of 2:1 by volume for formalin and tissue at room temperature and allowed to dry for 12 hours as recommended by Buesa (2012). Fixatives form covalent bonds with free amino acid groups in tissues, cross linking them so that cells were stabilized and locked in their current state and positions for correct interpretation. The freshly harvested organs were immersed into a solution containing 10% buffered formalin immediately after harvesting of the organs for purposes of stabilization of cells and maintenance of their structure. Formalin was buffered and osmotically balanced to act on the tissues in a manner that minimized shrinkage, swelling and autolysis and for improved permeability into all the cells. Fixation entails cessation of cell functioning in the tissue and to preserve the structure of the tissue in similar state to that of the living tissue. After fixation, 70% ethanol was used to remove excess water (dehydration) from the tissues and at the process minimized the possibility of putrefaction. During dehydration, water from within the tissues are replaced by alcohol.

3.13.2 Impregnation of Tissues

After clearing alcohol from the tissues using Xylene, tissues are transferred into molten paraffin wax for purpose of impregnation. During this process clearing agent diffuses out and molten wax is infiltrated. The wax which has infiltrated into the tissue is meant to fill the intracellular spaces and hold the tissues in place for further handling

3.13.3 Embedding of tissues

Embedding refers to enclosing tissues in a paraffin media and allowing it to solidify in specially designed plastic trays. Tissues and organs are soft and fragile, even after fixation, there is still need to be embedded in a supporting medium before being sectioned. The tissues in the trays were oriented in a manner that would allow them to be cut in a cross section appearance. Paraffin wax, which was used as the supporting media, was put on small trays and the tissues put therein and were allowed to cool to create a solid base that supported the sectioning of the specimen. During the fixation process, tissue water was replaced by ethanol, which was also later replaced by wax that held the sectioned tissue firmly in position as it solidified. The paraffin wax is replaced with Xylene, which serves as a clearing agent of the wax from the tissues.

3.13.4 Sectioning of tissues

This is the cutting of consistent paraffin embedded thin slices of tissue samples. The microtome block containing the microtome knife was used to slice tissues and the paraffin block mounted on the microtome carried the sample specimen having been pre-set to ensure the uniformity of the sectioned sample tissue. The tissue in the paraffin block was held by the microtome which moved this block towards a sharp knife in a rotary motion to cut the tissue into pre-set thin slides of 5 to 10 μm thick that was pushed forward inform of a ribbon. The slides were spread out on warm water bath and handled gently in an effort to avoid any possible formation of wrinkles, scratches or the loss of some parts. The quality of the sectioned tissues were assessed visually and the best of the slides were picked using a pair of forceps and placed on the slide. Then, the slide was labeled to indicate the name of the organ the tissue was taken from and date the slide was prepared. Having been laid on

glass slides, the tissue slides were placed on a rack for further processing and viewing. The slides were dried at 37^{o}C for 1 hour to gently melt the paraffin wax and leave the sectioned tissue intact.

3.13.5 Staining of tissue sections

The cellular components of tissues are normally clear and were made distinct from other components in the current study by the process of staining. Tissue sections were stained with dyes that have specific affinity for particular sub cellular components. The sectioned tissues were stained using hematoxylin and counter stained using eosin. Hematoxylin is basic with a deep purple or blue colour and works best with basophilic cells to give distinctive appearance. Eosin is an acidic stain with red colour and stains acidophilic cells.

The staining process started with dewaxing of the sample with hydrocarbon solvent-xylene for 20 minutes. The xylene on the slides was then removed using ethanol before rinsing thoroughly with water for 10 minutes. The slide was at this point hydrated to facilitate the penetration of aqueous reagents into the tissue samples slides. Following rehydration, the slides were stained using hematoxylin, that consisted of a dye and an Aluminum salt which serves as a binding agent in the solution. The hematoxylin imparts a red-purple or blue colour to the tissue in the slide. Further addition of a Sodium ion(Na+) alkaline solution gives the hematoxylin stained nucleus a dark -blue color. The slides at this point were rinsed with water, and checked for proper staining. For a case where the background was not clear, further bluing and thorough rinsing using xylene was carried out to achieve proper contrast of the background of the slide. Slide sections at this point were counter

stained using an aqueous solution of eosin for the purpose of colouring non-nuclear parts of the section with different pink colour. The section was rinsed using a solution of xylene to clear the tissue, dehydrate and make the sample clear and transparent for viewing.

3.13.6 Mounting of tissue sections

A layer of polystyrene mounting was applied, before applying the cover slip in readiness for microscopic viewing of the slides. The slides prepared from the block of fixed and stained tissue was mounted on glass slides and covered with thin glass cover slip where a transparent histomount (synthetic-clear mounting media) which hardens and seals the preparation to make it permanent was used. The cover slip was held at angle of 45° to the surface of the slide, then allowed the bottom edge to touch the drop of histomount and suddenly dropped to cover the tissue. The excess histomount was cleared from the slide and was ready for microscopy.

3.13.7 Histological Examination

The histological examination of the hypothalamus, anterior pituitary gland, ovary, uterus, fallopian tube and the liver were carried out using binoculars light microscope (Olympus CX2 Model, Japan) at magnification X 100. The microscope was fitted with a digital camera that was used to take photomicrographs of the observed histological status of the tissues and organs.

3.14 Morphological characterization of ovarian follicles

The follicles from various treatments were characterized physically to determine the effect of aflatoxin on their morphology among New Zealand white rabbits. Development and regression of follicles occur as a results of normal growth and/or structure and therefore it's important to identify follicles as to whether they are in morphologically stable state or undergoing atriesia. Morphological characteristics known for growing mammalian follicles are observed during pre-antral and primary follicular stages, which may be observed as columnar granulosa cells, cuboid or cuboid with ellipsoid end point of the antrum.

3.15 Statistical Analyses

The data collected from hormone quantity determination from laboratory analyses were entered into an excel program spread sheet of the computer and saved in a file ready to be imported for ANOVA analysis. The non-normal distributed continuous hormone levels data was transformed into normal distribution through log transformation formula;

(Logarithmic function is: **f (x) = k + a log b**

where a, b, k, and h are real numbers such that b is a positive number $\neq 1$, and $x - h > 0$.

A logarithmic function is transformed into the equation: f (x) = 4 + 3 log) .

In the log transformation, the variables were transformed into percentile rank which resulted into uniform probabilities, followed by the second step that employed the inverse-normal transformation of the percentile data into normally distributed z-scores before being

analyzed according Genstat (2008) at probability level of 0.05. When the P value was less than 0.05, then the null hypothesis was rejected and the alternative accepted. One-way ANOVA (Appendix 2 to 3) was carried out to determine whether the mean of the hormone levels differed significantly for the 16 rabbits in replications which were assigned to the four treatment groups of diets containing different levels of aflatoxin. Significant means were subjected to multiple comparison using Tukey test (Appendix 4 & 5) to separate the treatment means that showed significant ($P \leq 0.05$) differences.

3.16 The association between histological changes Vis-a-vis hormones levels

The data was subjected to non-parametric rank correlation to measure the strength of the relationship between the main variables in the study. The measurements used Kendall's Tau rank correlation coefficient, which assesses statistical associations based on the ranks of the raw data that is coded for purposes of analysis.

Before the analysis was carried out the histological changes were evaluated blindly(assigning codes to each level of damage) on the organs and scored based on descriptions of aflatoxin level-induced histological changes as described by Hoerr (2003). The changes scored included inflammatory reactions, cell necrosis, vacuolar degeneration and cell infiltrations on a scale of 0 to 3 scale, with 0 indicating no change (0 = no changes, 1 = mild lesions; 2 = moderate lesions; 3 = severe lesions) as described by Sridhar *et al*. (2014). Once the score had been completed, the average mean score of degeneration, cell infiltration and inflammatory changes were used for statistical analyses and

determination of the level of associations of hormone levels in plasma and histological changes of organ.

In the first instance, the association among oestradiol, follicle stimulating hormone and luteinizing hormones were studied. Secondly, the association between Oestradiol, FSH and LH Vis–a–Vis aflatoxin quantities in the diets were carried out. Then finally, the concentrations of the hormones were tested for association with the histological changes of the hypothalamus, ovary and liver were tested. The parameters considered for association in histology included inflammatory reactions, cell necrosis and leukocyte infiltration.

CHAPTER FOUR

RESULTS

4.1 Concentration of Hormones in plasma among rabbits fed diets laced with different levels of aflatoxin

Aflatoxin at all levels included in rabbit diets in the current study was found to have had significant ($P<0.05$) effect by lowering the concentration of oestradiol, follicle stimulating hormone and luteinizing hormone as shown in table 4.1 below;

Table 4.1: The amount of oestradiol, follicle stimulating hormone and luteinizing hormone in treatments of the study

Treatment	Number (n)	Oestradiol (pmol/l)		FSH (mlu/ml)		LH (mlu/ml)	
		Before the trial	Mean± SD	Before the trial	Mean± SD	Before the trial	Mean± SD
1 (Zero aflatoxin)	12	23.08	18.36± 7.12[a]	0.66	0.52± 0.43[a]	0.4	0.29± 0.07[a]
2 (100 ppb aflatoxin)	12	24.0	16.51± 6.52[ab]	0.93	0.38± 0.55[b]	0.78	0.21± 0.08[b]
3 (200 ppb aflatoxin)	12	22.16	11.35± 7.39[b]	0.71	0.23± 0.75[b]	0.3	0.19± 0.05[b]
4(400 ppb aflatoxin)	12	25.11	7.65± 5.91[c]	0.79	0.27± 0.64[b]	0.38	0.18± 0.12[b]
s.e			2.76		0.46		0.35
P-value			0.05		0.05		0.05
			*		*		*

when the quantity of aflatoxin were increased in treatments 2, 3 and 4, the concentration of oestradiol, follicle stimulating hormone and luteinizing hormone concentrations appeared as represented in bar graphs given in figures 4.1, 4.2 and figure 4.3 below;

The concentration of oestradiol hormone among treatments are shown in figure 4.1 below.

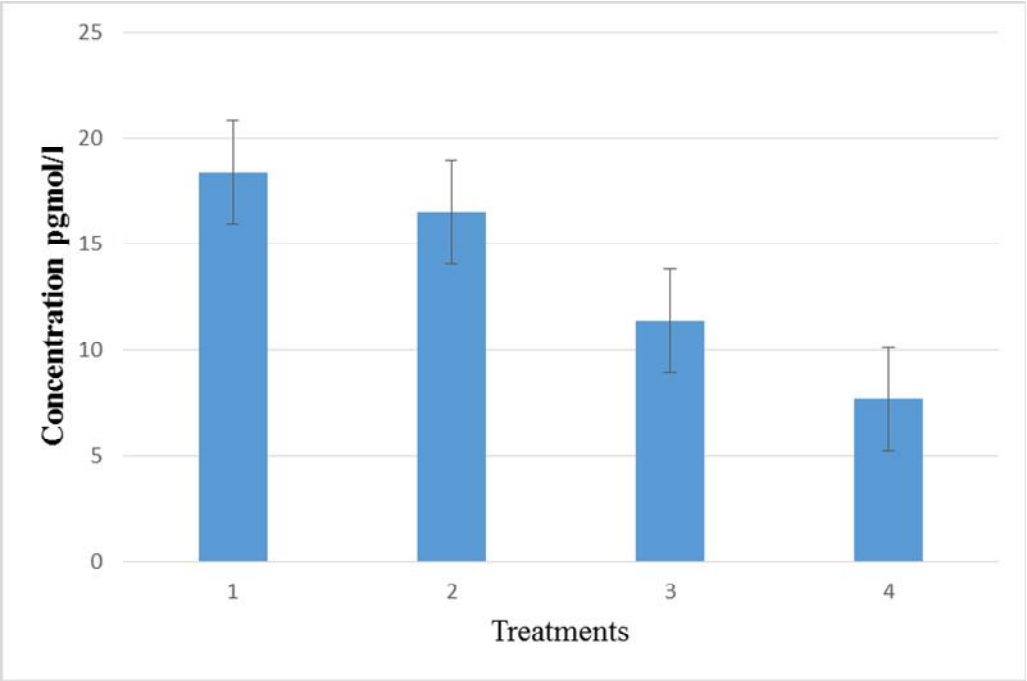


Figure 4.1: The oestradiol concentration in the four treatments

The experimental New Zealand white rabbits in the Control treatment had highly significant ($P<0.001$) different Oestradiol concentration in blood plasma compared to the rabbits in treatments 3 and 4. The latter two treatments involved the rabbits fed diets laced with 200 and 400 ppb of aflatoxin. The trend observed was indicative of reducing concentration of oestradiol at increasing amount of aflatoxin from treatments 2 to 4, to the

extent that, the mean concentration of oestradiol was highly significantly (P<0.001) lower in treatment 4 compared to control and treatment 2.

The post-hoc pairwise comparisons showed significant (P=0.002) difference between the control treatment and treatment 4 (with 400 ppb aflatoxin) and highly significant (P=0.0013) difference between treatment 2 (100 ppb aflatoxin) and treatment 4 (400 ppb aflatoxin).

Concentration of follicle stimulating hormone in the various treatments are given in figure 4.2

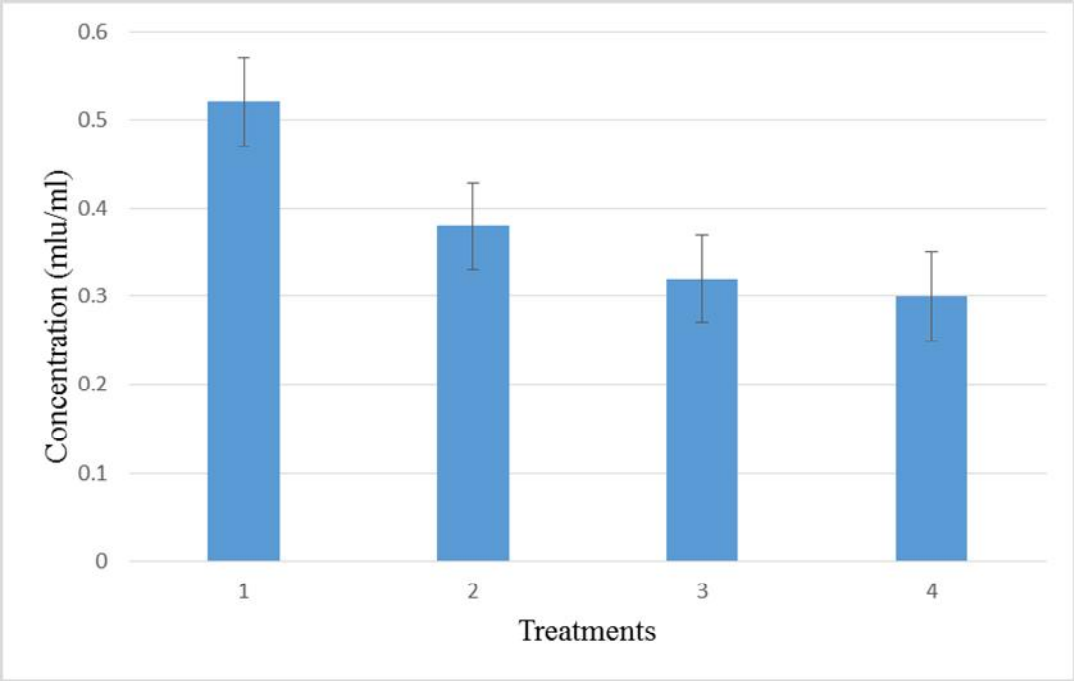


Figure4.2: The follicle stimulating hormone concentration in the treatments

Aflatoxin quantity in treatments had significant (P≤0.05) effect on follicle stimulating hormone concentration in plasma as shown in figure 4.2. The diet with unquantified level aflatoxin (treatment 1) (0.52±0.05) gave the highest FSH concentration than the diets with aflatoxin, but the lowest level was observed in treatment 4, that contained 400 ppb of aflatoxin at 0.25±0.07.

Concentration of luteinizing hormone in the various treatments are given in figure 4.3

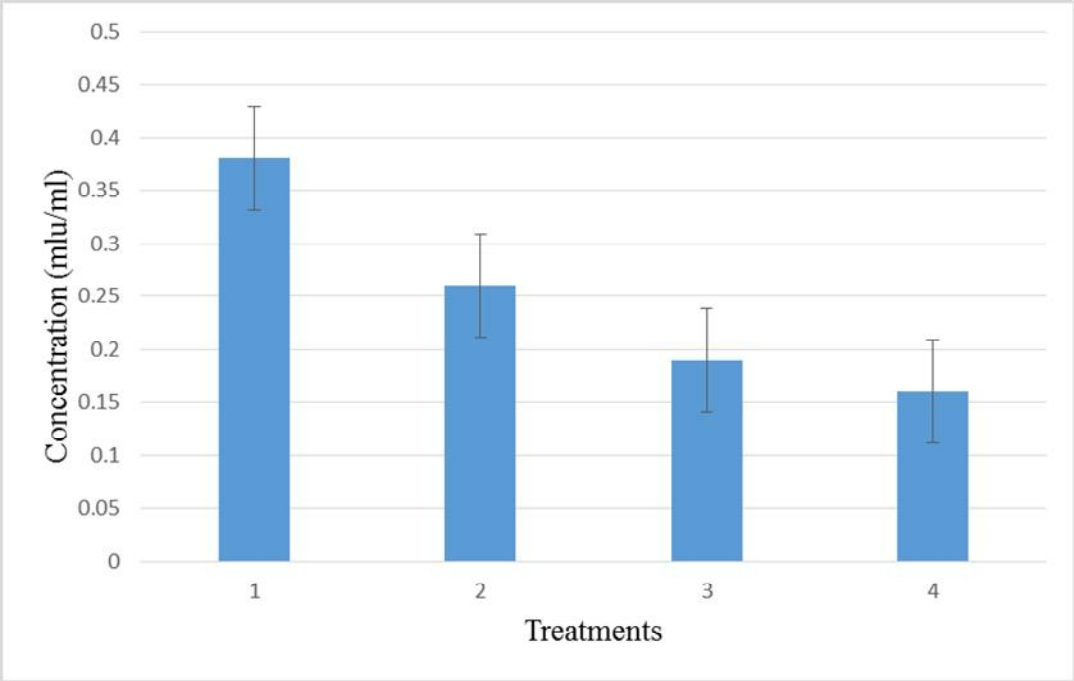


Figure 4.3: The luteinizing hormone concentration in treatments

The mean concentration of luteinizing hormones were highly significantly (P≤0.01) different in treatment 1 compared to treatments 2, 3 and 4, where the quantity in treatment

1 was higher than the latter three treatments. The mean concentration of LH in treatments 2, 3 and 4 did not significantly (P>0.05) differ when compared among themselves but showed a reducing trend with increasing aflatoxin level from treatment 2 to 4.

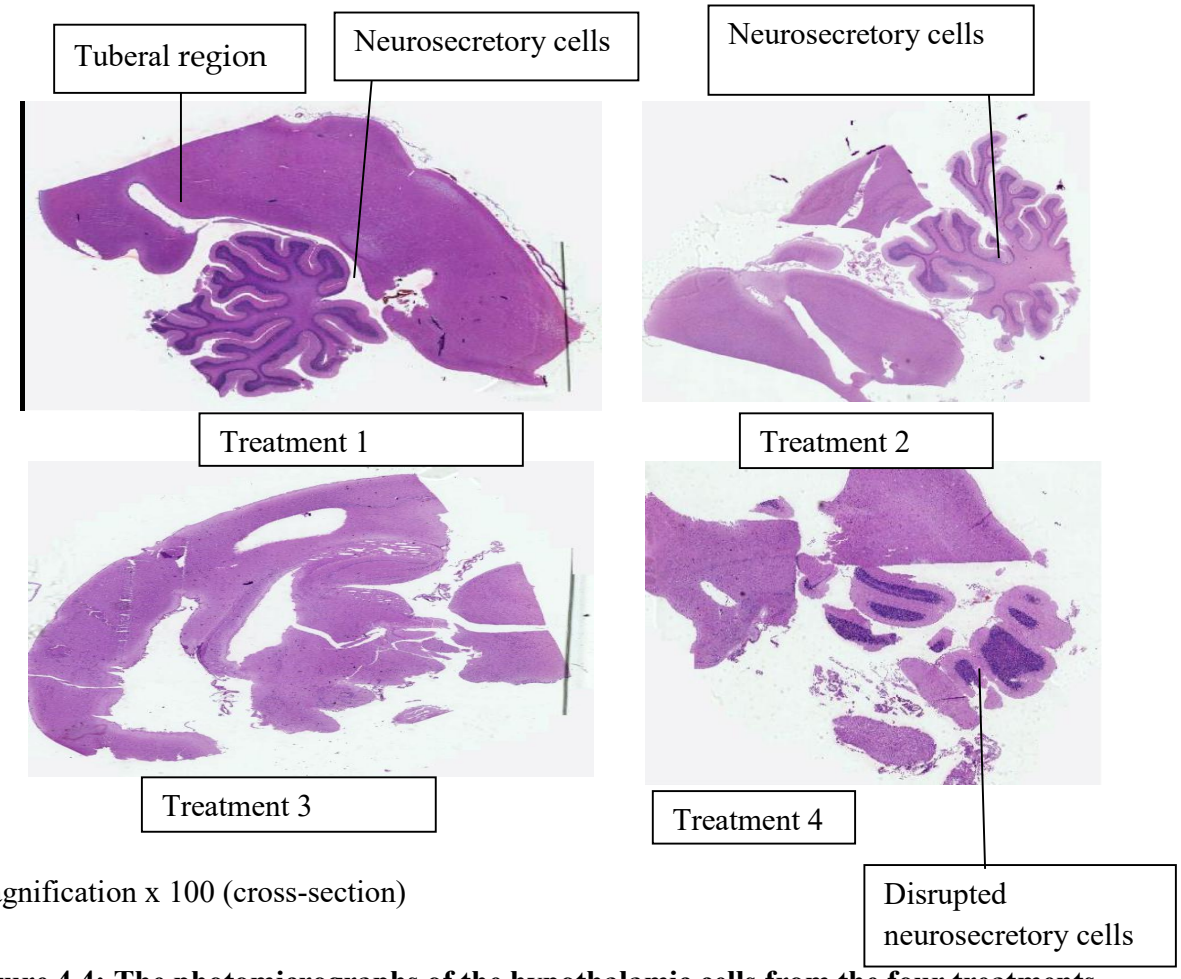
In the post-hoc pairwise comparison of LH concentration, the current study found that treatments 1 was significantly (P=0.043) different compared to the concentration in treatment 3(200ppb of aflatoxin). Luteinizing hormone was likewise found to be significantly (P=0.013) high in the control group than in treatment 4 (400ppb of aflatoxin). The study also found LH concentration to be higher in the control than in the treatments 3 and 4.

The study found high positive correlations among the hormone quantities of interest in the study. Oestradiol had a positive correlation of 0.79 with follicle stimulating hormone, while luteinizing hormone had a high positive correlation with follicle stimulating hormone of 0.8.

But the correlations between aflatoxin and oestradiol, follicle stimulating hormone and luteinizing hormone were 0.15, -0.04 and -0.06 respectively. These current relationships were quite low. The relationship of aflatoxin and follicle stimulating hormone and luteinizing hormone were low but negative.

4.2 The histology of hypothalamus, pituitary gland, ovarian, uterine and liver tissues in rabbits fed diets laced with different levels of aflatoxin

4.2.1 Hypothalamic histology in rabbits fed diets laced with varying levels of aflatoxin



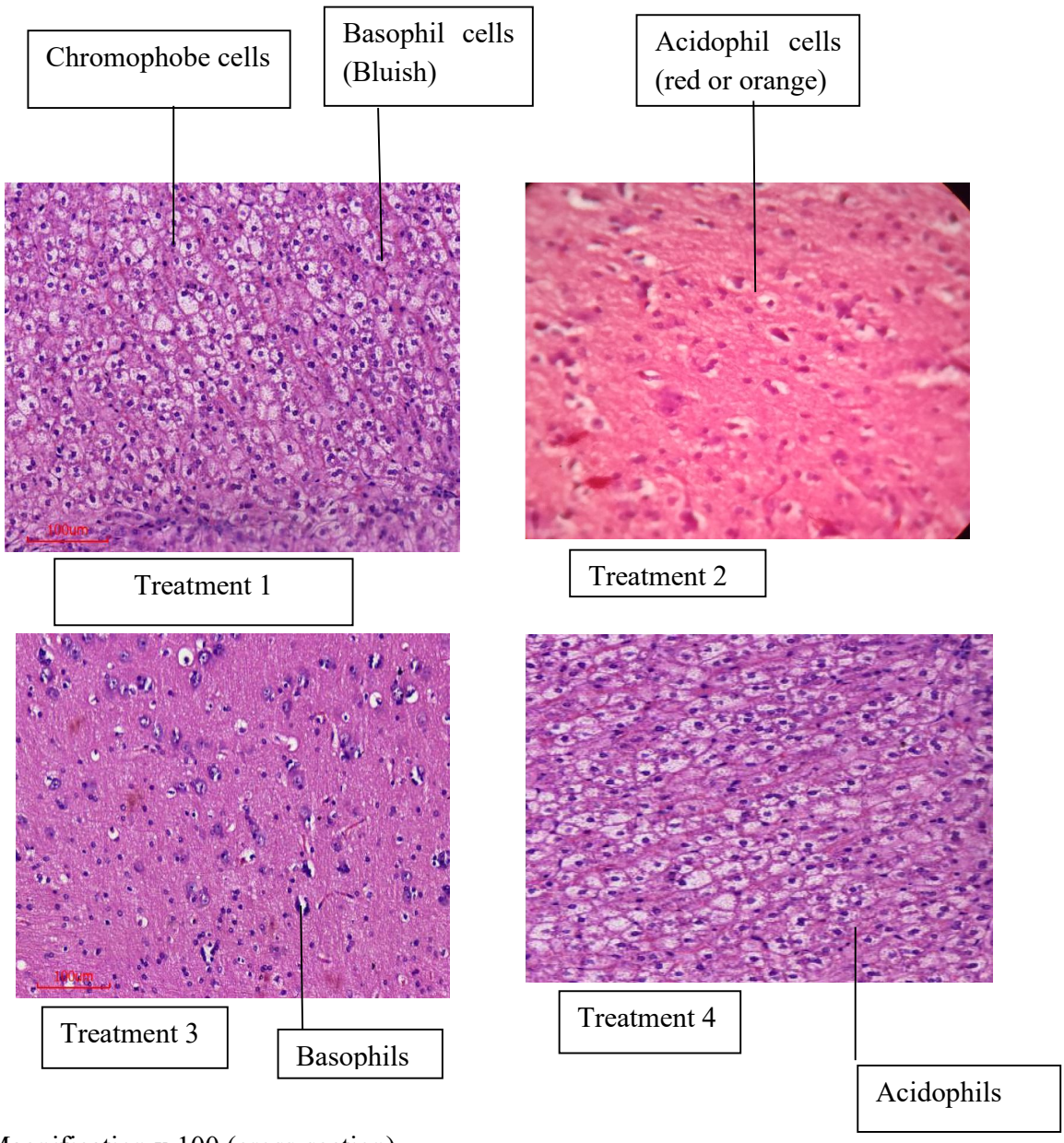
Magnification x 100 (cross-section)

Figure 4.4: The photomicrographs of the hypothalamic cells from the four treatments

Figure 4.4 shows the neurosecretory cells of the preoptic area of the hypothalamus. These neurosecretory cells were affected by aflatoxin consumption (treatments) by the rabbits. Alteration of the hypothalamic neurons were observed and indicated the toxicity on the hypothalamic neurosecretory cells. The neurosecretory cells appeared to be in the storage phase of the neurotransmitters state in treatment 1. They showed vesicles and dense granules which are indicative of neurotransmitters and endocrine cells to be used at the appropriate moment.

The storage phase of neurosecretory cells was indicative of functional status of the hypothalamus, when they were ready to secrete neurotransmitters for synaptic purposes and hormones which act as releasing or inhibitory factors.

4.2.2 The pituitary gland histology in rabbits fed diets with varying aflatoxin levels



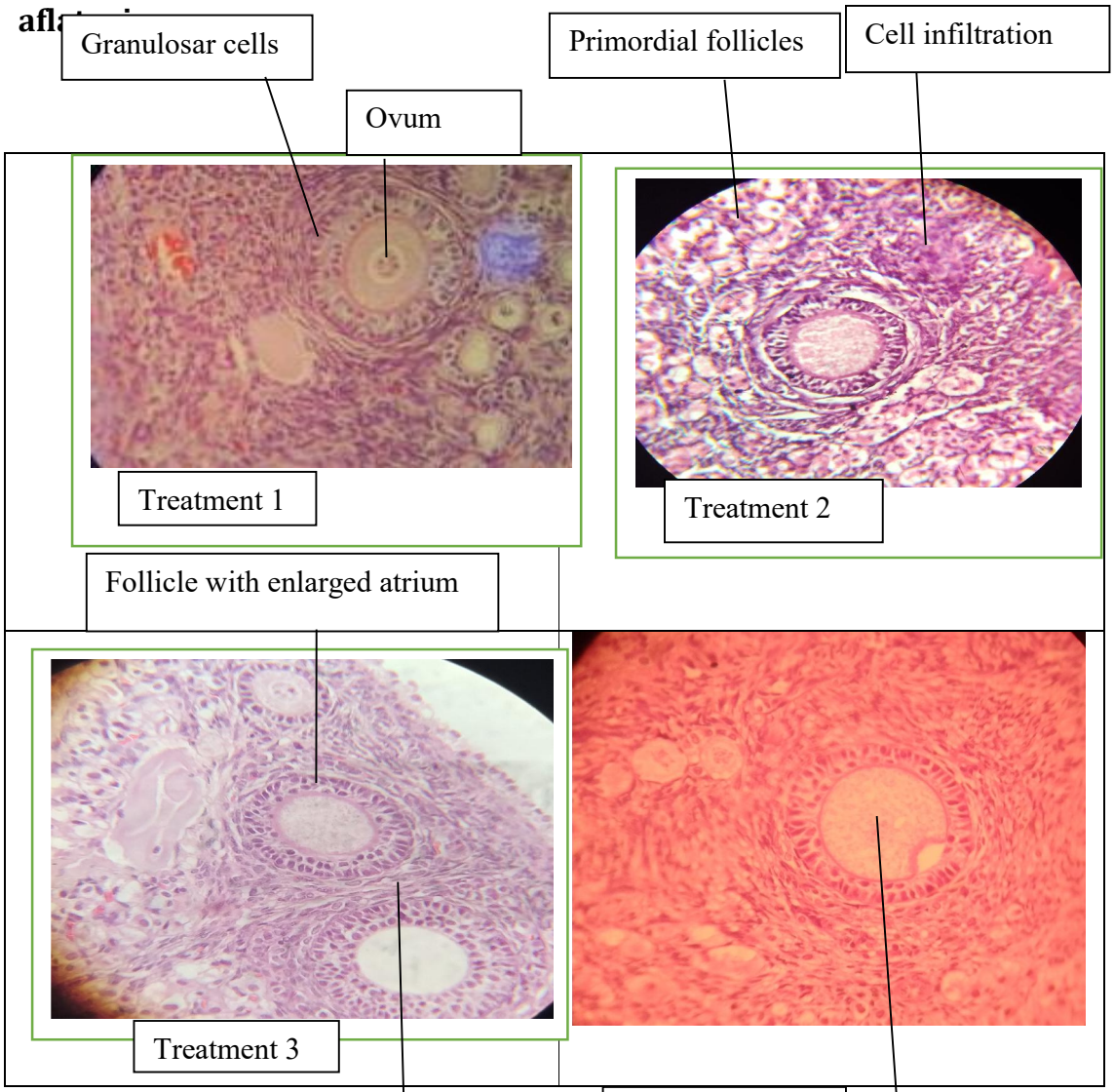
Magnification x 100 (cross-section)

Figure 4.5: The photomicrographs of the Pituitary gland histology from treatments 1,2,3 and 4

The pituitary gland in all the treatments in the current study showed chromophobe, acidophil and basophil cells. The histology of the pituitary gland in this study was not affected by aflatoxin at all levels of inclusion in the treatments as shown in figure 4.5. The pars distalis are secretory cells in the anterior pituitary gland that are divided into acidophils- showing red nucleus, basophils- pinkish in colour and chromophobes- shown in form of gray with clear surrounding. The basophils secrete gonadotrophic and thyrotropic hormones.

The photomicrographs in figure 4.5 showed that the acidophil, basophil and chromophobe cells were not histopathologically damaged by aflatoxin intake in the diets provided in treatments as shown in figure 4.5.

4.2.3 The ovarian histology in rabbits fed diets with varying levels of afla



Magnification X 100 (Cross section)

Figure 4.6: The photomicrograph of the ovary showing follicles and the aflatoxin effects in treatments.

The effects of aflatoxin on follicles is shown in photomicrographs given in figure 4.6 of the ovaries in all the treatments and the stages of development are shown in table 4.2.

Table 4.2: Follicles observed per treatment from figure 4.6

Treatment	Follicle	Size (µm)	Proportion within treatment (%)	Necrotic follicles	Necrotic (%)
1	Secondary	92.0[a]	16.5	0	0
	Primordial	22.8	84.5		
2	Secondary	90.1[a]	12.5	4	50
	Primordial	22.1	87.5		
3	Secondary	85.6[a]	66.7	8	66.6
	Primordial	18.8	33.7		
4	Secondary	78.4[ab]	8.3	8	66.6
	Primordial	17.6	91.7		

The photomicrographs of treatment 1 showed one secondary follicle and a total of five primordial follicles. The secondary follicle had two layers of columnar granulosa cells and a well-developed antrum. The treatments with aflatoxin showed fewer secondary follicles with less cuboidal shaped primary follicles and single layered granulosa cells. The study observed reducing follicular sizes with increasing aflatoxin levels as shown in table 4.2.

The secondary follicle size was significantly ($P \leq 0.05$) larger in treatment 1 compared to treatment 4.

In treatment 1, the ovary showed seven primordial and innumerable antral follicles whose appearance was similar to that of clustered grapes. The graffian follicle showed a well-developed oocyte and granulosa cells. The primary follicles in this treatment had poorly developed oocytes and granulosa cells.

In the photomicrograph of the ovary in treatment 2, shown in figure 4.6, the observable primordial follicles were seven. Part of the theca lutein cells were covered by dark - red blood- like fluid, which was an indication of congestion which, and may have been a manifestation of hemorrhage in the stroma or infiltration of leukocytes as an inflammatory reaction and congestion of the medulla due to the effects of aflatoxin. However, three necrotic follicles, which formed 50% of the total follicles in treatment 2 were identified. This study found ovoid primary follicles containing large antrum and pre-antral follicles with poorly developed granulosa and oocyte cells.

The photomicrographs of the ovary in treatment 3 as shown in figure 4.6 was indicative of a single primordial follicle but had two secondary follicles. The glandular cells around the secondary follicle showed slight congestion which could be an indicator of infiltration of either histamines or leukocytes, resulting into dark-red inflammatory area. The inflammation could have blared the visibility of other follicles. However, two follicles appeared necrotic. The morphological appearance of follicles in this treatment were a

single layer of columnar granulosa cells and enlarged antrum. One follicle appeared haemorhagic with shrinking antrum.

The primordial and primary follicles formed the majority in the glandular area of the ovary in treatment 4, where only one secondary follicle was observed. The morphological characteristics of the follicles in treatment 4 were as follows; there was one ovoid secondary follicle which had well developed single layered columnar granulosa cells. The primary and primordial follicles manifested enlarged antrum and shrunken granulosa cells. The zona pellucida was poorly developed in the primary follicles.

Histological examination of ovarian tissues in treatments 3 and 4 showed minor changes characterized by desquamation of granulosa cells, associated with congestion and multifocal oaedema in between congested blood vessels in the ovarian medulla. Large numbers of luteal follicles compared to the number of secondary follicles were observed. The examination of the sections revealed the presence of vacuolar degeneration and necrosis within the corpus luteum, accompanied by hyperplasia of the interstitial cells in between the lutein cells. Further examination of the slides of treatment 4 showed hyperplasia of the interstitial cells associated with hypertrophy of the medulla.

The secondary follicles in treatment 4, showed enlarged atrium and single layers of cuboid granulosa cell. A single growing follicle manifested the multiplication of cuboid granulosa cells.

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4.2.4 The uterine histology in rabbits fed diets with varying aflatoxin levels

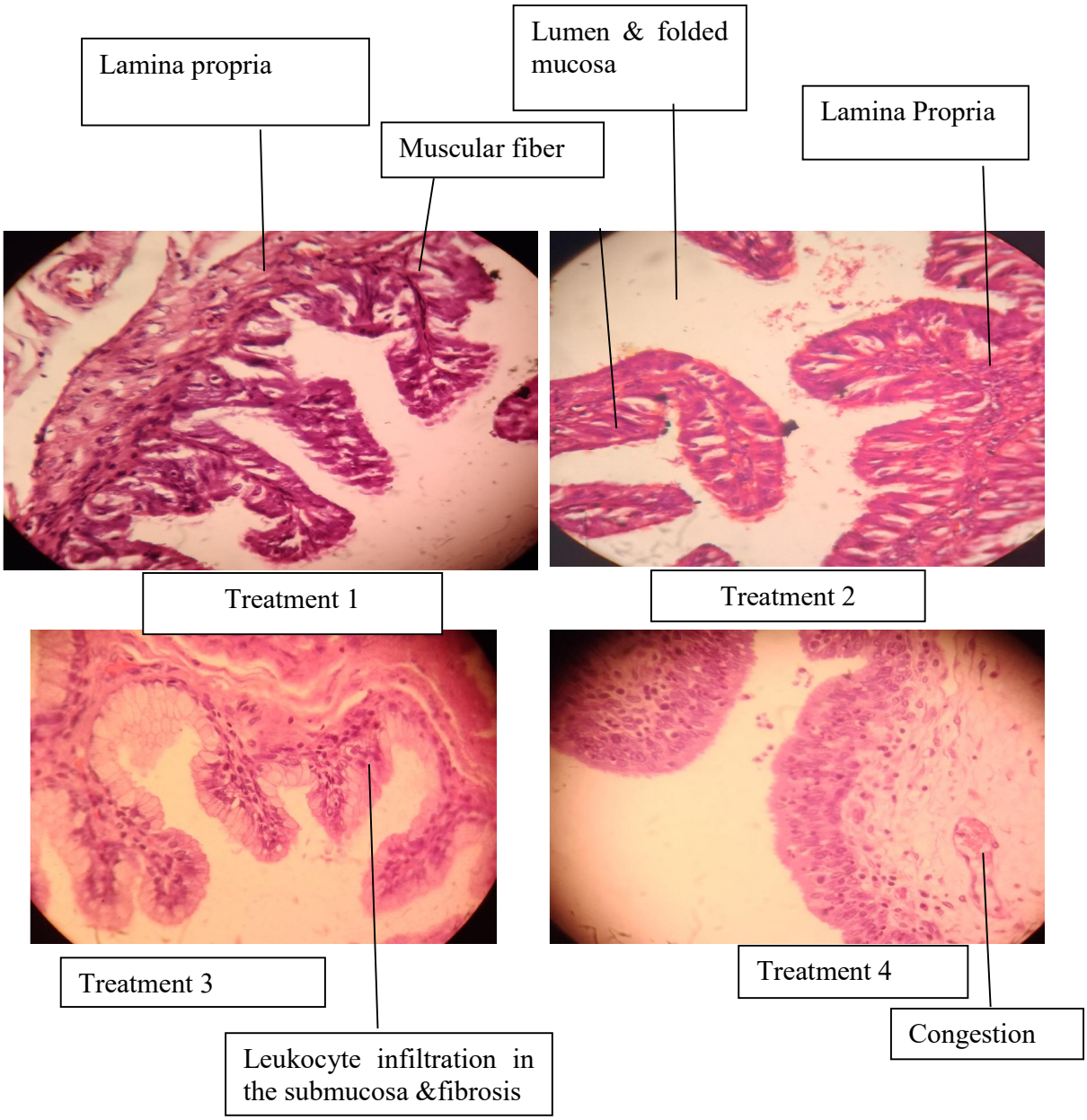

Figure 4.7a: The photomicrograph showing the cross section of the uterus (Magnification X100)

The uterus from treatments 1 and 2 as given in figure 4.7a showed anatomically normal histological structure with minimum aflatoxin's pathological effects. There were minor focal aggregation of acute inflammatory neutrophil cells in the lamina propria in the submucosa of the endometrium, observed in treatment 2. The features of the cells in treatments 1 and 2 as observed appeared productive as indication of the uterus' proliferative stage.

The uteri in treatments 3 and 4 revealed marked necrosis of the lining of both the epithelium and the endometrium. Degeneration of the lining of epithelium of the uterine glands, appearing as vacuoles with mild leukocytic cell infiltration into the submucosa and lamina propria, and focal necrosis of lamina epithelialis were observed. Congestion of the myometrial blood vessels and massive fibrosis of the lamina probria were noticed in some instances as shown in figure 4.7b.

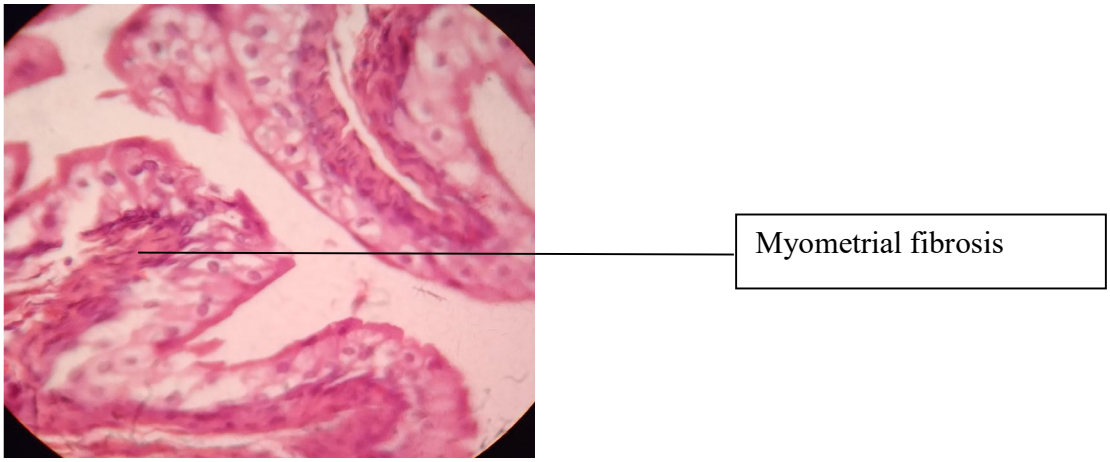

Figure 4.7b: The photomicrograph showing uterine tissue with fibrosis (Magnification x100)

4.2.5 The fallopian tube histology in rabbits fed diets laced with aflatoxin

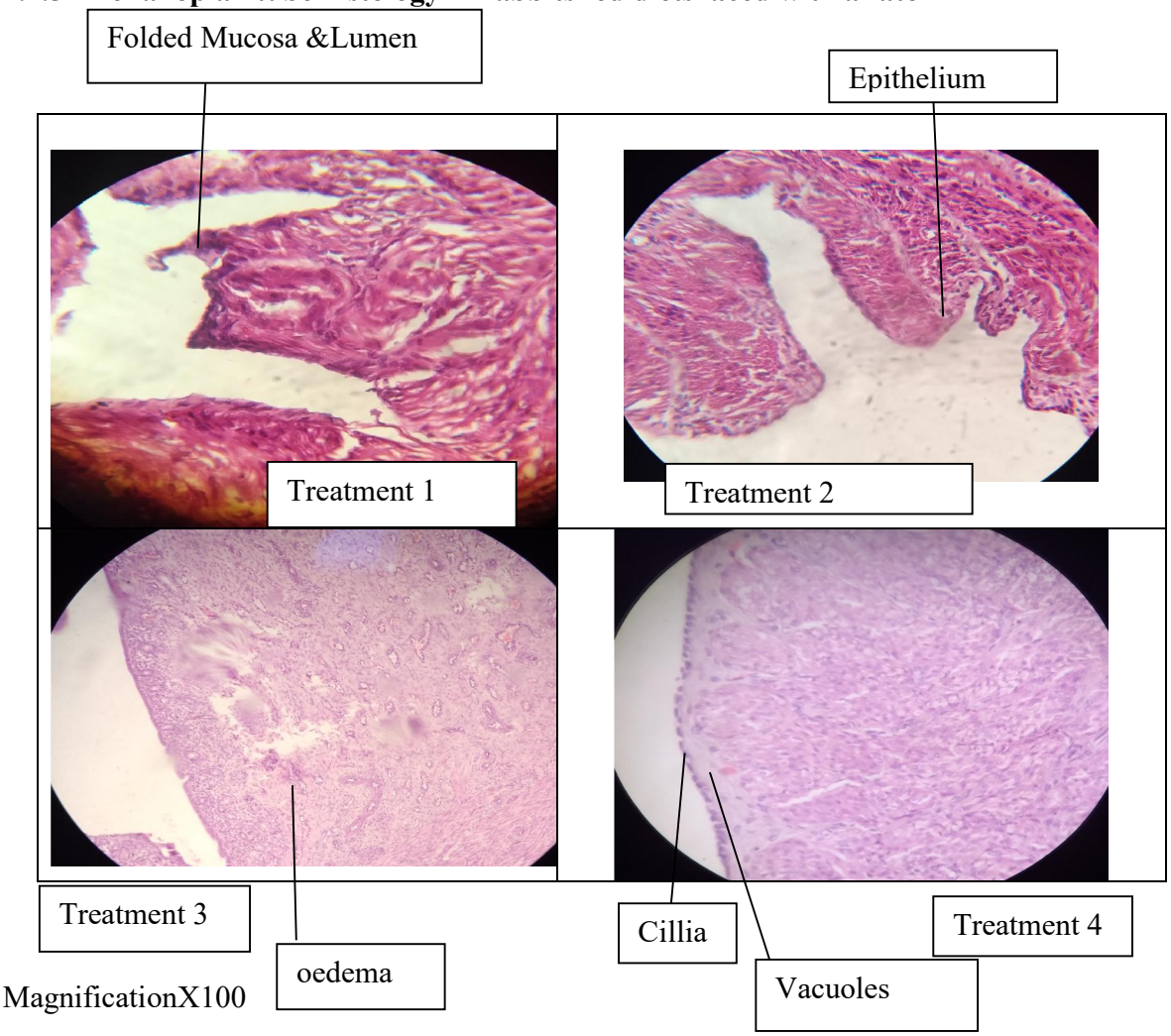

Figure 4.8a: The photomicrograph of fallopian tube indicating folds and vacuoles.

Examination of the cross-section of the fallopian tubes given in figure 4.8 showed vacuolar and inflammatory effects in treatments 3 and 4. These defects manifested as vacuolization of the lining of the epithelium of the endometrium.

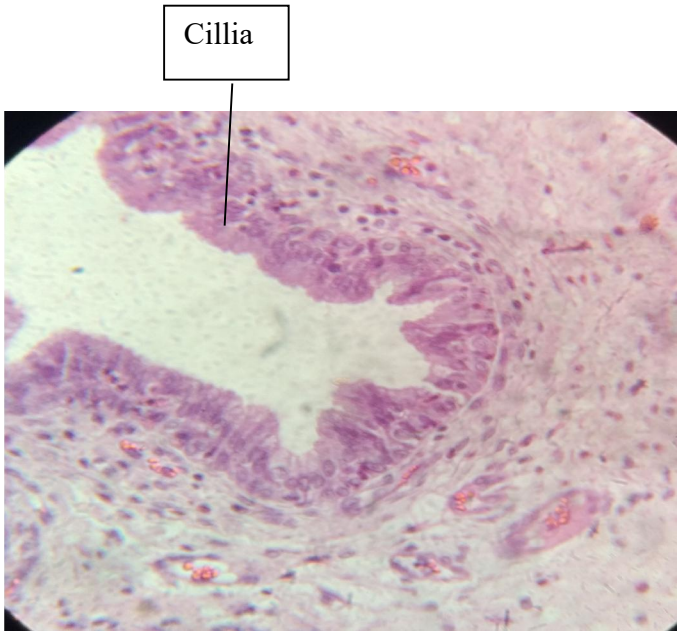

Figure 4.8b: The photomicrograph of fallopian tube cross section indicating a mash of cillia.

4.2.4 The Liver histology in rabbits fed diets with varying aflatoxin levels

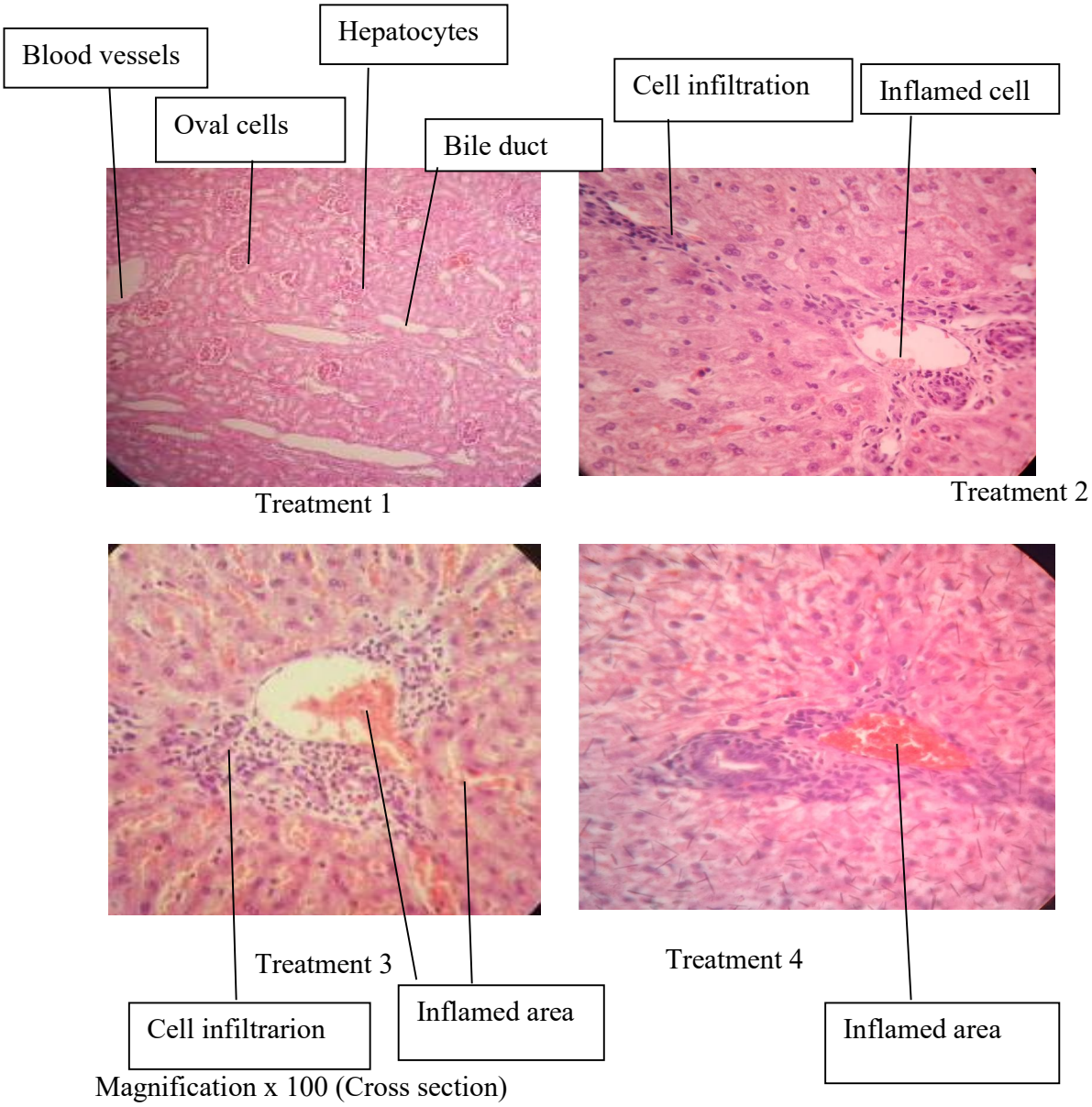

Figure 4.9a: The photomicrograph of liver-periportal area obtained from rabbits in all the treatments.

The photomicrograph in figure 4.9 show the periportal area of the liver with various components and level of effects by aflatoxin. Treatment 1 showed hepatocyte cells, oval cells, blood vessels and bile ducts in an anatomically normal and functional state.

The photomicrograph of the liver cells in treatment 2 as indicated in figure 4.9a, showed inflammatory reactions to aflatoxin. Focal inflammatory area are an indication of lesions visible in the periportal area of the liver, which are indications of damage to the liver hepatocyte cells. The dark spots are an indication of influx of probably lymphocytes in response to the effect of aflatoxin.

The photomicrographs of the liver cells shown in treatment 3 and 4 of figure 4.9a was indicative of inflamed areas showing vascular degenerative changes of the hepatocyte cells and congestion of the blood vessels. Hepatic injury shown in figure 4.7b was observed as periportal inflammatory cell infiltration, bile duct proliferation, fatty acid changes and dilated central veins indicated in figure 4.9b. Infiltration of lymphocytes and indicative inflammatory cells around the peripheral and oval cells showed congestion of sinusoid and may limit the proliferation of vessels and bile ducts.

The samples from treatments 2, 3 and 4 were affected though the latter showed higher infiltration and inflammatory damage, but the rabbits in treatment 1 showed no cellular damage.

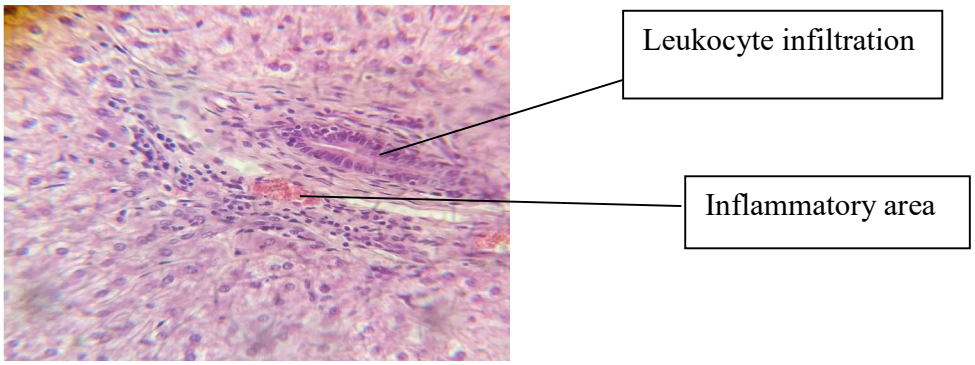

Figure 4.9b: The photomicrograph of the Liver hepatocyte in treatment 4 showing both inflammation (red spot) and Leukocyte infiltrated area (dark-blue

4.3 The association between hypothalamus, pituitary gland and gonadal tissue changes and hormones levels

Table 4.3: The correlation coefficients of histopathology of the liver and hypothalamo-pituitary-gonadal tissues Vis-a- Vis hormones levels in plasma

	Treatment (aflatoxin)	FSH levels	LH levels	Oestradiol levels	Hypothalamus tissues changes	Pituitary tissue changes	Liver tissue changes	Ovaries tissue changes
Treatment	1.00							
FSH levels	-0.12*	1.00						
LH levels	-0.30*	0.77*	1.000					
Oestradiol levels	0.152	0.38*	0.276	1.000				
Hypothalamus tissues changes	0.36*	0.115	-0.14	-0.04	1.000			
Pituitary tissue changes	0.215	0.057	-0.07	0.07	0.590*	1.000		
Liver tissue changes	-0.695*	0.39*	0.44*	0.35*	-0.48*	-0.48*	1.000	
Ovary tissue changes	-0.663*	0.39*	0.44*	0.39*	-0.47*	-0.48*	0.98*	1.00

The results in table 4.3 showed that hormone levels significantly ($p<0.05$) correlated negatively with the treatments. The Follicle stimulating hormone showed low negative correlation with aflatoxin ($\rho = -0.120$). The luteinizing hormone had a significant ($p<0.05$) negative association with the quantities of aflatoxin of -0.298 as contained in treatments. But oestradiol did not show any significant ($p>0.05$) association with the treatments ($\rho = 0.152$). The association between hormone level and aflatoxin in this study was quite low. The aflatoxin content showed positive significant $p<0.05$) association to hypothalamic tissue changes of 0.358 and significant ($p<0.05$) negative association to liver tissue changes of -0.695 as well as to ovary tissue changes of -0.663 as indicated in table 4.3.

The oestradiol, follicle stimulating hormone and luteinizing hormone concentration had significantly ($p<0.05$) low positive association with the liver and ovary changes as shown in table 4.3. There was significant ($P\leq0.05$) low correlations between aflatoxin content in the diet vis-à-vis liver and ovary tissue changes as shown in table 4.3.

CHAPTER FIVE

DISCUSSION

5.1 Concentration of Hormones in plasma among rabbits fed on diets laced with aflatoxin

The concentration of oestradiol in the current study showed reduction in treatments with with increasing aflatoxin level to below optimum hormone quantities reported for mammals, possibly due to damage of hepatocytes or follicles resulting into inhibition of fat metabolism and bringing about decreased synthesis of cholesterol, precursors for oestradiol synthesis (Handan & guleray, 2005). The reduction in oestradiol in the current study followed the trend reported by El Mahady *et al*. (2015). Hasanzadeh *et al*. (2011) reported lower amounts of oestradiol of 4.5 p/ml as compared to the current study may be because of the fact that the researcher fed higher levels of aflatoxin (0.8 to 3.2 ppm), used cattle animal species (ruminant), which could have been affected by rumen microbial enzymes and the specific species of Cytochrome Phosphate 450 of the heifers. Kourousekos *et al*. (2015) reported declining quantities of oestradiol with time of aflatoxin consumption. This implied that longer time of exposure or higher intake could bring about higher effects on oestradiol concentration compared to short time exposure. Hasanzadeh *et al*. (2011) reported the decline of oestradiol when rats consumed more than 3.2 ppm of the aflatoxin but did not observe any reductions at 0.8 ppm. Oetradiol secretion occurs in healthy follicles whenever FSH initiates the action of aromatase enzyme in the granulosa cells of

the follicle and uterine epithelial cells leading to the secretion of oestradiol due to positive action of oestrogen receptor alpha (ERα) (Stocco & Anderson, 2019).

The hormone levels in the current study were generally affected by the amount of aflatoxin which ranged from 100 – 400 ppb in the diet and therefore were in accordance with Gupta's (2011) generalized assertion that blood hormone concentration were lower for aflatoxin fed animals than those which were exposed to limited amounts. This agreed with Marin *et al*. (2013) who reported that animal exposure to aflatoxin have been found to result into deleterious effects on female reproductive system through interference with the biosynthesis, metabolic process or reduced quantities of steroid hormones, the down grading of steroid receptors or interruption of oocyte maturation and its competence (Kourousekos *et al*., 2018). Aflatoxin has been known to induce endocrine reduction due to its effects on the expression of aromatase enzyme (Storvik *et al*., 2011). This author categorized aflatoxin as an endocrine disruptor, which has the potential of interfering with steroid hormone quantities. Reduction of oestradiol hormone quantities during the follicular phase could result into deleterious effect on the fertility of an animal (Kourousekos *et al*., 2018).

Aflatoxin has been reported to affect the synthesis of steroid hormones from the placenta by disrupting the enzymes involved in its biosynthesis, metabolism and conjugation, therefore interfering with foeto-placenta hormone balance (Huuskonen *et al*., 2013). It may also affect the functioning of the follicular cells or the hypothalamo-pituitary-ovarian axis

(Ibeh & saxena, 1997). The current findings showed different trends to those reported by Mahady *et al*. (2015), who despite using higher doses of aflatoxin of 250, 500 and 1000 ppb on heifers, did not find any difference in hormone levels, could be due to the fact that he used ruminants which have microbial enzymes playing a role of degradation of major molecules including aflatoxin in the rumen before absorption.

Oestradiol is a multifunctional hormone on several tissues among male and female animals, whose actions are mediated through the cytoplasm and nuclear of protein receptors ER_{α} and ER_{β}, which are responsible for starting signals that are either genomic or non-genomic in nature either to start the reproductive process or stop it accordingly (Korach *et al*., 2018). Low oestradiol concentration is necessary for the production of GnRH from the hypothalamus, which in turn is responsible for the secretion of gonadotropic hormones from the anterior pituitary gland to initiate ovulation in the animal (Jadav *et al*., 2010).

Hormonal control of oestradiol secretion is typically under the control of hypothalamo – pituitary - ovarian hormone regulation and feedback mechanism (Hamilton *et al*., 2017). Levels in the serum are monitored and regulated in the hypothalamus by neurons, through nuclear oestrogen receptors (ERs) which activate negative feedback controls whenever serum oestradiol is more than the amount required for physiological homeostasis (Hamilton *et al*., 2017).

The hypothalamus releases the GnRH in a pulsatile manner, which activates the secretion of the gonadotropic hormones from the anterior pituitary. The luteinizing hormone activates the ovary's theca and granulosa cells in readiness for rapture, at the same time,

follicle stimulating hormones triggers the granulosa cells to initiate the synthesis of steroid hormones. Increases of oestrogen beyond the physiologic limits inhibit oestrogen hormone synthesis via the oestrogen receptor alpha-mediated control of gonardotropin releasing hormone release and subsequent reduction of pituitary gonadotropin secretion (Hamilton *et al*., 2017). Arising disruption on such negative feedback control pathway may end up in hormonal disorder (Hamilton *et al*., 2017).

The gonadotropin releasing hormones secreted from the hypothalamus and transported to the pituitary gland is stimulated into production in response to spontaneous rhythms and sensory impulses from sensory inputs derived from the external and internal environments of the animal (Goncalves *et al*., 2010). Aflatoxin effect of internal body organs in the current study, which included inflammation and damage to ovaries, uterus and the neurosecretory cells at the hypothalamus may be the possible reason for reduced release of gonadotropin releasing hormone secretion and thereby could have affected gonadotropin hormone secretion from the pituitary gland. In the case where aflatoxin has caused inflammation of macrophages and neutrophils around the follicles as found in the current study, the production of oestradiol, which gives feedback to the hypothalamus, affect reproductive behavior and indirectly ovulation, could easily have interfered with reproductive performance of the rabbits. A functional hypothalamus, could have responded to positive feedback from oestradiol to secrete more GnRH, leading to high release of FSH and LH which was not the case.

The follicle stimulating hormone functions by causing ova maturity while luteinizing hormone facilitates the trigger that culminates in ovulation and discharge of the ovum from the Graffian follicle of the ovary in the animal that has attained oestrus phase or when it has been mated in the case of rabbits.

Jensen *et al.* (2004) and Hasanzadeh *et al*. (2011) reported increased follicle stimulating hormone production in female rats consuming aflatoxin and Hasanzadeh *et al*. (2011) attributed this to the destruction of the germinal epithelium that produces inhibin, which under normal situations, controls the production of follicle stimulating hormone. According to Jensen *et al*. (2004), serum follicle stimulating hormone becomes higher whenever the germinal epithelium is damaged causing the lowered circulating inhibin in the blood. But Amin *et al*., (2019) reported a decline in follicle stimulating hormone among buffalos fed on diets containing aflatoxin. In the current study, quantities of follicle stimulating hormone were significantly reduced to 0.3 mlu/ml for the treatments with aflatoxin, but did gave an indication of reducing trends with increasing aflatoxin quantities and this may be an indication that inhibin was in circulation as stated by Jensen *et al*.(2004). Follicle stimulating hormone in synergy with androgen hormone are responsible for initiating the synthesis of Aromatase enzyme that causes the secretion of oestradiol from the follicular granulosa cells and uterine epithelium.

There was no much effects of hormone quantities among rats consuming diets with aflatoxin of doses between 0.8 to 3.2 ppm as compared to control (Hasanzadeh *et al*.,

2011). This was an indicator that aflatoxin might have been acted upon by enzymes that lead more to hydroxylation than formation of reactive oxygen species (ROS) and therefore did not deter hormone production. But still, Hasanzadeh *et al*. (2011) and Amin *et al*. (2019) observed a decline in luteinizing hormone with increasing level of aflatoxin and hypothesized that any damage on the GnRH generator or anterior pituitary gland may result into reduced production of luteinizing hormone among animals or direct destruction of this hormone in plasma circulation. On the other hand, tumors on the hypothalamus have been reported to cause blockage of neurons and may prevent GnRH flow into the pituitary gland and therefore prevents the secretion of LH (Tunbull & Rivier 1999). Aflatoxin has also been reported to have a hypophysotoxic effect on animals, which is responsible for the lower secretion levels of both FSH and LH (Clarke *et al*., 1987; Hasanzadeh *et al*., 2011; Amin *et al*., 2019). The hypothalamus is required to release the GnRH in a pulsatile state for it to activate sufficient secretion of the gonadotropic hormones from the pituitary gland. Low levels of oestrogen in plasma triggers the secretion of LH while the rising levels oestrogen causes more secretion of FSH

Negative energy balance in animals provoke low reproductive activity because the body shifts to catabolic actions and less supply of energy for metabolic functions leading to compromised secretion of reproductive hormones. Metabolic fuels regulate hormone input to GnRH neurons, leading in turn to GnRH release and the appropriate drive via the hypophysis to the gonads (Evans & Anderson, 2012).

The negative correlation between luteinizing and follicle stimulating hormone production and aflatoxin found in this study, implied reducing hormone secretion with increasing aflatoxin quantity over a longer time, but this reduction was not statistically captured for the hormone concentration in plasma neither was any physiological reproductive behavior observed. This however was not indicative as to whether it would affect pulsatile levels (particularly for LH) required to initiate oestrus occurrence in an animal. The luteinizing hormone concentrations have been found to be lower at 0.34 mlu/ml in aflatoxin-fed birds and laboratory animals, resulting into delayed maturity among both male and female animals (Lakkawar *et al*., 2004).

Reports indicate that increased aflatoxin levels in the diet of farm animals resulted in their decreased fertility(Kabar *et al*., 2006). This has been attributed to aflatoxin's oestrogenic structure which has negative effects and damage on the main organs responsible for reproduction in the animal's body (Kabar *et al*., 2006; Zheng *et al*., 2005).

Energy intake among animals has been reported to have greater influence on LH release although restrictions may not stop its release, but too low amounts in LH store might not bring about the attainment of pulsatile state to effect hormone secretion (Short & Adam 1988). Short & Adam (1988) reported glucose as the only energy source utilized by the neural-endocrine system that is involved in the control of hormone secretion and has positive influence on the reproductive behavior of animals. This author further stated that blood glucose concentration is the specific determinant of energy intake for reproduction.

Compromised nutritional status decreases pre-ovulatory follicular functions that may result into low oestradiol concentrations, probably due to low cholesterol supply into the mitochondria for steroidogenesis (Verma, 2004). Verma (2004) further found undernutrition to suppress female reproduction through reduction of GnRH secretion from the hypothalamus, delay of onset of puberty and commencement of oestrus cycle. Reduction in intake of dry matter, could imply reduction in availability of glucose for animals, because the bulk of feed is an energy source which when metabolized will provide the required glucose to provide power for neural and endocrine functions.

Reproductive problems and retained placenta, among other factors, in lactating animals has been attributed to high aflatoxin levels of 3.0ppm in the animal's diets (Pirestani & Toghyani, 2010). This was also thought to cause increased negative energy balance, impairment of hormone production and inflammations in the placenta, factors which have negative effects on the animals reproduction (Ozysoy *et al.,* 2005).

Aflatoxin, has been shown to interrupt with the functioning of different endocrine organs through disruption of enzymes and substrates concerned with the synthesis of different hormones. Aflatoxins, its metabolite and the generated reactive oxygen species have been known to cause cancer in some endocrine glands including pituitary gland, granulosa cell tumor in the ovary and adenocarcinoma of the adrenal gland, thyroid gland, ovaries, parathyroid glands and pancreas (Lakkawar *et al*., 2004;AAFRD 2003; Butterworth, 2000).

Two unrelated toxic actions have been suggested to explain the aflatoxin effects on doe fertility; direct antagonistic interaction with steroid hormone receptors due to structural similarity between the aflatoxin and steroid hormones and/or indirect effect on the dam mediated or induced hypo-vitaminosis A (Ozysoy *et al.,* 2005). Metabolic fuels, on the other hand, that regulate inputs to GnRH neurons, leading in turn, to altered gonadotroph release to the gonads is also an important action, because GnRH drives the pulsatile secretion of LH particularly, from the pituitary gland (Evans & Anderson, 2012).

The findings of the current study included the effect of aflatoxin at the levels used on the amount of oestradiol, follicle stimulating hormone and luteinizing hormone, contrary to what was reported earlier that all quantities of aflatoxin manifest similar effects on all hormones (FAO1997).

5.2 Effects of aflatoxin on the hypothalamus, pituitary gland Ovary and Uterus in rabbits

5.2.1 Hypothalamic state in rabbits fed diets laced with aflatoxin

The neurosecretory cells of the hypothalamus were observed to have been affected by aflatoxin consumption (treatments 4) by the rabbits in the current study. The lesions observed in the hypothalamic neurons were indicative of the toxicity of the hypothalamic neurosecretory cells. Studies have shown that rats exposed to 100ppb of AFB1 resulted in significant alterations in chemical factors that are indicative of neurotoxicity, and this was

reported to have shown the degeneration of the central and peripheral nervous systems (Ikegwuonu, 1983). According Trebak *et al*. (2015) decreased manifestation of hypothalamic neuropeptides among rats fed diets having 150 ppb and 300 ppb of aflatoxin B1 disrupted the regulation of neuropeptides in the hypothalamus and affected the release of GnRH.

Aflatoxin, its metabolites and products that include reactive oxygen species, particularly AfB1-8, 9-epoxides have been reported to interfere with the functions of the nervous system by reacting to form DNA adducts, amino acid adducts, oxidative stress factors, mitochondrial indicative apoptosis of nerve cells and inhibit the biosynthesis of proteins, RNA and DNA (Liu & Wu, 2020). All these are factors that can counteract the proper functioning of the hypothalamus.

The hypothalamus controls the functioning of the pituitary gland, which in turn controls gonadal functions and eventually reproduction (http://www.wisegeek.org), among other functions. The neurosecretory cells secrete releasing or release-inhibiting hormones that controls the pituitary gland in its production of gonadotropin hormones.

Aflatoxin in the current study affected the histology and possibly the synaptic chemical aspect of the hypothalamus through its corrosive nature. Hypothalamic dysfunction rarely occurs among animals, but whenever it occurs it involves swellings which cause inflammations (Tunbull & Rivier, 1999).

Repeated exposure of animals to high quantities of aflatoxin has been found to result in reduced body weight gains and has been attributed to its disruption of the hypothalamic regulation of neuropeptides that are involved in feeding behaviour (Trebak *et al*., 2015). Rats consuming aflatoxin have been reported to have reduced concentration of neurotransmitters; dopamine and serotonin by 37 and 29% respectively (Coulombe & Sharma, 1985). Disruption of either the hypothalamus or anterior pituitary gland by chemical substances may result into failure of reproduction among animals. Tumors on the hypothalamus may cause blockage of the neurons and could prevent GnRH flow into the pituitary gland and therefore preventing the action which was intended to be performed (Tunbull & Rivier, 1999). The current study did not observe any tumor but lesion on the hypothalamus that could be attributed to toxicity by aflatoxin at the levels included for the study.

The delay in oestrus among the young stock and prolonged resumption of cyclicity among multipparus animals consuming aflatoxin contaminated diets, could be due to failure in cellular function of endocrine organs, glands and their production levels of oestradiol, follicle stimulating hormone and luteinizing hormone are possible other factors that affect reproduction. This is worsened by any histopathological damage on the hypothalamus where it may interfere with the secretion of GnRH. The pituitary gland may also be interrupted so that its secretion of gonadotropin hormones is sub optimal or the damage of follicles and the epithelium of the uterine endometrium thus causing low production of

oestradiol which is necessary for feedback to the hypothalamus to secrete GnRH. Organ and tissue damage leads to reproductive dysfunction, low secretion of hormones which lowers fertility among rabbits.

The consumption of aflatoxin contaminated concentrate feeds, among other factors, is responsible for the lowered reproductive efficiency among domestic livestock in the recent years (Dochi *et al*., 2010). The amount of aflatoxin in these diets, particularly where farmers manufacture their own animal feed diets without using aflatoxin binders, may be among the contributing factor.

5.2.2 Effects of different levels of aflatoxin in diets on the Pituitary gland

The pituitary gland cells appearance in the current study was not affected by aflatoxin as observed microscopically in histological sections. Earlier work on aflatoxin showed that the immuno-pathology of this organ was intact, that is, not affected (Hatori *et al*., 1991). A study of the distribution of aflatoxin in organs by Christou & Tigas (2018) revealed its lowest concentration of 10 ppb in the hypothalamus and pituitary gland. The anterior pituitary gland is critical to reproduction as it produces the follicle stimulating hormone and luteinizing hormone, which in turn are responsible for changes in the ovary, maturation of follicles and release of fertile ova. Aflatoxin has been reported to affect reproduction by interrupting the synthesis and metabolism of steroid hormone receptors, and cause adverse effect on oocyte development and maturation (Regiane *et al*., 2013). According to Norton (2007), animals experiencing infertility are most likely to be deficient of these hormones.

Aflatoxin, which has been known to affect the expression of aromatase enzyme may have indirectly affected FSH and LH in the current study (Storvik *et al*., 2011).

The anterior pituitary gland has secretory cells in the pars distalis which are divided into acidophils, chromophorbs and basophils of which, the latter secretes the gonadotrophs. The population of these cells is supplied with abundant capillaries and their function is to secrete gonadotrophic hormones from sufficient supply of blood and food nutrients (Pineda, 1989). The pars distalis cells function according to the phase of their secretory cells. The pituitary cells enter into an actively synthesizing phase in response to increased demand for a particular tropic hormone as influenced by oestradiol feedback mechanism. The basophil cells secrete follicle stimulating hormone and luteinizing hormone, whose target organ is the ovary for maturation and rapture of follicles (Pineda, 1989).

Gonadotropic releasing hormone (GnRH) stimulates the release of follicle stimulating hormone and luteinizing hormone from the anterior pituitary gland. Low-frequency GnRH pulses are responsible for the release of FSH, whereas high frequency pulses cause LH secretions (Holesh & Lord, 2019). The pulse frequencies are determined by the number of GnRH receiptors in the hypothalamus. Follicle stimulating hormone initiates the growth and maturation of immature oocytes into mature follicles before ovulation, before the LH triggers ovulation (Holesh & Lord, 2019). The LH surge also, increases intra-follicular proteolytic enzymes, weakening the wall of the follicle to allow the mature ovum to pass through and be ovulated (Dosouto *et al*., 2019). The levels of aflatoxin used in the current

study of up to 400 ppb, had no change on the histology of the pituitary gland but had effects on gonadotrophic hormone secretion, which could have occurred indirectly through its effect on the feedback mechanisms or directly through its deformation of the hormones in plasma.

The current study found that aflatoxin at the levels used had no deleterious effect on the pituitary gland, though the levels of follicle stimulating hormone and luteinizing hormone were reported to to have decrease in quantity. The concurred with other findings among animals that consumed diets with similar levels of aflatoxin (Storvik *et al*., 2011).

5.2.3 Effects of aflatoxin laced diets on the histology of the ovary among rabbits

The findings of current study included degenerated follicles and inflamed theca lutein cells. There were fewer Graffian follicles observed, while the available ones and granulosa cells were shrinking in diets laced with aflatoxin as compared to the control.

The Ovaries of aflatoxin treated female rats with 500 ppb, were found by El Mahady *et al*. (2015) to have been damaged and the degree was dependent on the dosage of aflatoxin. The study by Shapour and Saeedeh (2013) found ovaries, manifesting intense atrophy, decreasing number of intact ovarian follicles in comparison to the appearance of multiple corpora lutae in the control treatment, and these agreed with the reports that animals predisposed to high aflatoxin than acceptable levels bring about histological effects on ovarian follicles at primordial follicular stage, particularly to those at pre-ovulatory stages (Regiane *et al*., 2013). Aflatoxin has been reported to diminish viability of oocytes by

disrupting their maturation through epigenetic modification and cause oxidative stress, high autophagy and apoptosis (Liu *et al*., 2015).

Follicular atrecia is a major loss of an oocyte which could have led to the fulfillment of the reproductive function and production in an animal. There are reports indicating that aflatoxin causes coagulative necrosis among primordial and secondary follicles and reduction in the number and sizes of primordial follicles as it increased the number of atretic follicles (Cauli, 2010 & Bemeur *et al*., 2010). Slow expansion of follicular antrium compared to the rate of granulosa cell multiplication has been reported to result into morphological alteration due to compacted granulosa cell layers that leads to the formation of columnar cells (Rodgers *et al.*, 2001).

Acute inflammatory response triggered by tissue injury involves the coordinated delivery of leukocytes to the site of injury (Barton, 2008). Aflatoxin is an exogenous inducer of inflammation which the body detects and mimics the virulent activity of pathogens, but the body relies on expulsion and clearance mediated by mucosal-epithelia cells resulting into infiltration of tissues (Barton, 2008). Aflatoxin has been reported to cause histological alterations in form of coagulative necrosis among primordial and Graffian follicles, decrease in the number and size of the follicles (Bbosa *et al*., 2013; Marai & Asker, 2008). This was observed in the current study due to fewer secondary follicles and reducing follicle sizes with increasing aflatoxin content in the diets. These observations were indicative of the apoptotic effects of the aflatoxin on the ovary as it has been reported to

cause deleterious damage to the reproductive capability among female rabbits through reduction in ovary and uterine size, foetal resorption, loss of conceptus through failed implantation and intra-uterine mortality among female rats (Bemeur *et al*., 2010).

Primordial follicles can be lost through atresia or apoptosis in ways that is not understood, but high chemical factors or DNA induced follicular atresia are possible causes (Nguyen *et al*., 2018). The great majority of follicles are known to be atrietic at particular stages of development due to their high level of sensitivity to chemical substances (Sonigo *et al*., 2018). Reports exist where chemotherapeutic drug exposures have resulted into reduced number and sizes of primordial follicles in women, which was associated to direct damage to follicular population (Meng *et al*., 2014; Le *et al*., 2017). Aflatoxin has been shown in the current study to have reduced follicles as was the case with drugs among women. However, direct chemical damaging effects of aflatoxin on primordial follicles *In vitro,* where, accelerated apoptosis and atresia has been observed (Yuksel *et al*., 2015).

Aflatoxin has been reported to impair the reproductive performance of female animals, where female rats showed significant reduction in the number of oocytes and the graffian follicles (Ibeh & Saxena, 1997). High dose exposure of 1000 ppb to aflatoxin has been found to cause reduction in ovarian size and increased foetal resorption among some animal species, including rats (Gupta, 2012).

The idea of categorized control of granulosa cell multiplication and follicular liquid containment has been useful in explaining two follicular phenotypes (Rodgers *et al*., 2001;

Irving-Rodgers & Rodgers, 2000). These categories among bovines can be separated based on morphology and activity criteria. The first phenotype shows the 'loopy' basal lamina while the other manifests the columnar basal granulosa cells and manifestation of the RNA unit of telomerase in the antral located cells (Irving-Rodgers & Rodgers, 2000; Lavranos *et al*., 1999).

It was hypothesized that in the event that the follicular antrum expansion is at a low rate compared to the replication of the granulosa cells, the cell layers become crowded resulting into more of the basal layers of granulosa cells changing into columnar shape (Rodgers *et al*., 2001). The concept connecting basal granulosa cell shape and follicular basal lamina phenotype to the speed of follicular antrum growth agreed with the finding that columnar basal granulosa cells and loopy basal laminas are not found in follicles that have grown beyond the dominance size or where the acceleration of antrum growth is reported to be dominant by ultrasonography (Irving-Rodgers & Rodgers, 2000; Irving-Rodgers *et al*., 2002).

Oocytes from follicles containing loopy follicular basal lamina cannot sufficiently stimulate replication of granulosa cells, resulting to loopy state of the follicular basal lamina (Rodgers *et al*., 2009). Loopy follicular basal lamina has been reported to be an indicator of poor quality oocytes (Irving-Rodgers *et al*., 2009). The low oocyte quality is associated with slow growth of follicles, which are thought to be due to its prolonged

period before activation and this may be an indicator or an earlier sign of follicular atresia before the actual cell death (Irving-Rodgers *et al*., 2009).

The reported delay in the occurrence of oestrus cycle among young and delayed cyclicity of lactating animals is probably due to aflatoxin inhibition of organ function, where it compromises cellular integrity because compounds that destroy or impair growth of ovarian follicles can have marked effect on attainment of puberty, onset or recurrence of oestrus cycle (Hasanzadeh & Amani, 2012).

Microscopic examination of the ovaries in the current study showed necrotic alterations in the follicular theca lutein cells, which was a possible indication of degeneration and atretic follicular damage by the aflatoxin. The number of follicles in the ovary of treated rabbits were lower compared to the control, an occurrence attributed to the intake of aflatoxin laced feeds. This concurred with the finding of Hasanzadeh & Amani (2012) who reported that increased consumption from 5.0 to 65.2 of aflatoxin in an animal's diet resulted in significant reduction of the number of healthy primordial, primary, secondary and tertiary follicles which affects the fertility of the animal. According to Wei-Hong *et al*., (2017) aflatoxin exposure affects the fertility of animals through the reduction of litter/brood size when he fed *Caenorhabditis elegans* diets containing 10, 30, and 90 μM of aflatoxin and recorded significant decrease in brood sizes.

Exposure of the rabbits to toxic compounds affect its fertility by either interfering with the GnRH, FSC or LH hormones as they are transported from the hypothalamus or pituitary

gland (Blank *et al*., 2007; Li *et al*., 2017). Aflatoxin can, likewise cause the total failure of the ovary by destroying the follicles, though it can also disrupt the neuroendocrine feedbacks resulting into an abnormal plasma level of follicle stimulating hormone and Luteinizing hormone (Blank *et al*., 2007).

The theca lutein cells gave a blurred indicator of vacuolar damage, probably due to inflow of histamines, extravasation of leukocytes and fluids released into the tissue, which coincidentally resulted into a dark-red inflamed appearance on the left hand side of the follicle in treatment 4. The follicles showed degenerative state possibly due to acute effects of aflatoxin. The follicle could therefore have been pathologically affected and therefore turning necrotic or a high flow of leukocytes or lymphocytes as a physiological reaction intended to manage the aflatoxin, which might have been effected by the control enzymes to counter its damage.

The Uterine structures of the female rabbits in treatments with aflatoxin in the current study showed marked histopathological changes manifested as necrosis of the epithelium of the endometrium. These showed greater damage compared to what Abd El-Wahab (1996) observed as ulceration when he fed 0.15 mg afB1/ kg body weight to rats. The same researcher reported degenerative changes in the uterine gland at much higher aflatoxin dose of 7.0 mg / kg body weight. After examining the uterine tissues El Mahady *et al*. (2015) observed mild hyperplasia of epithelial cells lining endometrium and lining epithelium of endometrial glands. The same authors also found Mild focal aggregation of mononuclear

inflammatory cells mainly neutrophils in the lamina propria in the submucosa of endometrium were seen.

The endometrium is the most specialized, active portion of the uterus that give responses to hormonal signals and initiates actions that lead to prolactin production in non-pregnant animals. Both oestradiol and progesterone are responsible for preparing the endometrium for possible implantation of the foetus incase conception has taken place (Shaw *et al*., 2010).

The reason for the differences in the intensity of damage could be attributed to different animal species and the family or species of cytochrome P450 enzymes that metabolized the aflatoxin in the liver, the length of time of exposure and the quantity of aflatoxin. Other reports show reduction in uterine sizes, increased foetal resorption and uterine foetal death among some animal species, including rats, when exposed to high doses of 220 ppb of aflatoxin (Gupta, 2012). The uterus is known to put up defensive actions for survival and recover through the cell infiltration observed in the current study in the submucosa of the endometrium and may include the action of macrophages and T cells in an effort to wade off damaging effects of aflatoxin.

The fallopian tubes are located on either side of the abdomen and they are also referred to as oviduct and are funnel tube-like structures, which serve as channels for the transportation of ova from the ovary to the magnum, where they are fertilized before the embryo is moved by the propping action of oxytocin hormone on smooth muscles and

moved to the uterus for implantation. There were some effects at the stroma portion of the fallopian tube, which could be associated with invaded cells due to the effects of aflatoxin. Vacuolization at the stroma-edge of the fallopian tube just after the cilia, affects fat components of the cell, this portion is associated with the synthesis of hormones for endocrine function.

Vacuolization refers to a change in morphology of animal cells following an infection by either bacteria, virus or contamination with substances of low molecular weight such as exudates from molds or by substance which are chemical in nature. Reports indicate the involvement of septin in processes associated with intracellular vacuolization in membrane related phagosomes (Hacker, 2018). Membrane vacuole development appears to be linked to any infection. The vacuolization of the stroma epithelium portion caused by aflatoxin consumption in the current study, risked the functioning of the smooth muscles, the cilia that facilitate the transportation of the ovum from the ovary, whereby, it may either hasten its movement due to the possible inflamed nature of the epithelium or cause a functional cessation. The ciliate function is controlled by oestrogen and progesterone hormones under normal functional status of the animal, and they initiate ovum mobility under the regulation of cilia and supported by the peristaltic action of the rhythmic contraction of the fallopian tube smooth muscles (Nutu *et al*., 2009). Low levels of oestrogen in plasma could also hinder the working of the cilia while Prostaglandins have likewise been found to upgrade the contractility of smooth muscles, thus facilitating the beating action of cilia and eventual transportation of ovum (Waggren *et al*., 2008). Reproduction among rabbits can be fully

functional if both the physical and endocrinological health status are guaranteed through the consumption of aflatoxin free-nutrient balanced diets.

5.3 Effects of aflatoxin laced diets on the histology of the Liver of rabbits

The liver is composed of 80% hepatocytes, which form the major parenchymal cells whose functions include nutrient metabolism, detoxification of poisonous compounds and the synthesis of amino acids which eventually form the protein component of the animal's body. They also capacitate body immune system against invasion by micro-organisms through secretion of immune-globulin proteins. The liver is a very important organ in all animal systems with multifunctional roles including metabolism and waste product elimination and is party to most biochemical pathways to growth, defensive actions against infections, nutrient provision, energy supply and reproductive activities (Ward & Dally, 2002; Bemeur *et al*., 2010).

Hepatocyte growth factors have been linked to the proliferation and regeneration of the endometrium and therefore are related to the involution of the uterus following parturition (Sugawara *et al*., 1997). It is the organ with the highest activation of estrogen receptors in an animal's body, but is dependent on the intake of sufficient amount of amino acids by the animal, which is necessary for the activation of the receptors (Torre *et a*l., 2011). Sufficient amount of estrogen receptors, activate the secretion of oestrogen receptor alpha (ERα), which expresses itself in the uterus and ovary to cause the release of oestrogen and synthesis of Insulin-like growth factor 1(IGF-1), where the former activates the

hypothalamus to release GnRH, that targets the anterior pituitary gland for the release of gonadotrophs (Tang *et al*.,2019). The IGF-1 on the other hand, activates the synthesis of growth hormone that causes the development of the uterine epithelium in preparation for the implantation of the conceptus (Torre *et al*., 2011, Tang *et al*., 2019). Insufficient amount of amino acid intake by animals, results into the production of ER_{β} which degrades the secretion of oestrogen and therefore oestrus cycle and any reproductive process in animals is terminated (Tang *et al*., 2019).

Toxicity of dietary aflatoxin in mammals has been reported to cause damage to various organs of which the liver is the prime target (Azab *et al*., 2008). It has been recognized to cause serious health problems among animals, where following the early discovery of aflatoxin, FAO (2010) reported that it induces hepatic carcinoma at levels as low as 15 ppb. In the current study, aflatoxin affected the liver at all levels of inclusion in the diet. The cumulative effect of aflatoxin arising from intake in diets contributed to gradual deterioration of hepatocytes, which in the long run may affect the overall health and fertility of the consumer (Frank *et al*., 1994). Liver degenerative lesions have been observed, which at times appear mild or to a severe degree in test treatments, at times showing parenchymal degenerative characteristics of granular appearances of hepatocyte cytoplasm, though the majority of hepatocytes manifested cytoplasmic visualization with necrotic cells among groups with interventions (Ward & Dally, 2002).

Any liver damage that brings about failure in its function renders it unable to detoxify ammonia, products of amino acid metabolism bringing about hyperammonemia that can

pass the blood brain barrier resulting to an increase in the synthesis of glutamate neurotransmitter that brings about the toxicity of brain cells whose result is encephalopathy (Bemeur *et al*., 2010 & Hussain *et al*., 2008). Aflatoxin has been reported to bring about colour change and enlargement of the liver, congestion of parenchyma, cytoplasmic vacuolation or hepatocytic fatty changes and necrosis and there are reports of mononuclear and cell infiltration among broiler chicks (Bemeur *et al*., 2010).

Aflatoxin has been reported to affect reproduction through interruption of synthesis, metabolism and interference with the steroid receptors, adverse effect on oocyte development and maturation (Regiane *et al*., 2013). The effect on hepatocytes in the current study could be deleterious for reproduction particularly when it affects the receptors and oocytes and may be worst if the animal consumed it over a longer period causing chronic effect arising from consistent deposition on target organs. Inflammatory reactions in the liver or ovary could affect estrogen secretion and /or estrogen receptors, which may result into infertility among rabbits. It has been found that any abnormal function of oestradiol synthesis is correlated to endocrine diseases and endometritis, and at the same time, fertility reductions have been reported among women and animals with liver diseases (Tang *et al*., 2019 & Pu *et al*., 2020).

Aflatoxin has been reported to induce oxidative damage through the generation of free radicals which react with other cellular components in the body causing pathological changes in the liver functions (Peles *et al*., 2019). Toxins that affect protein components of this organ affect enzyme production, particularly CYP450 family which is produced from

the liver and kidney cells. In their findings on mice, Yassein & Zghair (2012) observed low levels of this enzyme on damaged liver following consumption of aflatoxin. The enzyme is important for the detoxification of toxic complex compounds and its absence could result into degeneration of hepatocyte, advancing in chronic form into cancerous state whenever there is continued exposure to aflatoxin.

The degeneration of liver cells, periportal inflammatory cell infiltration and bile duct proliferation observed in the current study concurred with the findings of Yassein & Zghair (2012) who observed lesions characterized by vascular degeneration in the cytoplasm of the hepatocytes with some cells showing apoptosis. Yassein & Zghair (2012) observed an aggregation of macrophages but scattered liver parenchymal cells around the central vein and fatty changes in the cytoplasm of the hepatocytes, an indication of the dissolution effects of aflatoxin on the fats, thus affecting its consistency.

Periportal necrosis observed in the current study, was indicative of the direct uptake of the aflatoxin through the blood stream into the liver. FAO (1997) reporting on electron microscopic radio autography on aflatoxin treatment observed that a single hepatic dose, leads to the aflatoxin binding onto the liver hepatocyte cytoplasm more than it does in the nucleus.

The liver is a non-endocrine organ which synthesizes certain hormones and hormone precursors and at the same time play a vital role in the regulation of hormone balance in an animal's body. Cellular hepatic damage, could inhibit enzyme actions or lipid and fatty

acid synthesis necessary for gonadal or gonadotropin hormone biosynthesis (El Mahady *et al.,* 2015). Among other important hormones, it synthesizes insulin like growth factor 1, which is important in the regulation of cell growth and development through the stimulation of growth hormone. On the other hand, about 80% of cholesterol, a molecule necessary for the formation of steroid hormones, is produced in the endoplasmic reticulum of the hepatic cells of the liver (Regiane *et al*., 2013).

5.4 The association between aflatoxin, hypothalamus, pituitary gland-gonadal tissue changes vis-a-vis hormones levels

The current study found negative correlation between follicle stimulating hormone and luteinizing hormone Vis-a-Vis the aflatoxin amount in rabbit diets. This was indicative of the deterrent effects aflatoxin has on the secretion of the two hormones. The findings agreed with those of Amin *et al*. (2019), who reported significant reduction of these two hormones among buffalos that consumed diets containing 21.2 ppb of aflatoxin.

Though aflatoxin had no histological effect on the pituitary gland, the FSH and LH concentrations were found to have declined in was an indicator of negative effect it has on the reproductive system finally. This showed that consumption of increasing quantity of aflatoxin by animals led to the reduction of follicle stimulating hormone and luteinizing hormone. Aflatoxin has been reported to affect reproduction by interrupting with the synthesis and metabolism of steroid receptors, and cause adverse effect on oocyte development and maturation (Regiane *et al*., 2013). Regiane *et al*., (2013) reported that an injection of luteinizing hormone to aflatoxin consuming chicken did not improve

reproductive activity while the controls had positive change caused by the injection of the hormone. This imply that aflatoxin cause dysfunctioning of LH in plasma of rabbits. There are reports also that silent pituitary adenomas interrupt with the secretion of gonadotropic hormones, by either the down grading of receptors or a small number of receptors are functional at the pituitary gland (Drummond *et al*., 2019).

The low association of follicle stimulating hormone and luteinising hormone Vis-a-Vis the hypothalamus and pituitary gland changes could be explained by the fact that the pituitary gland did not show histological damage and yet the hormones showed low concentration in plasma. Though the negative insignificant association of LH to both the hypothalamus and the pituitary gland was indicative of the fact that in a situation of increased organ change, the LH would diminish in concentration.

The non existence of significant correlations between oestradiol and the hypothalamus, and the pituitary gland could be indicative of the fact that the hormone is anyway not secreted by any of the two organs and could have only influenced the secretion of GnRH which was not put into perspective in the current work.

Significant positive association was observed between the hormones oestradiol, follicle stimulating hormone and luteinizing hormone to the liver and ovary damage by aflatoxin. The association was positively manifested because higher necrotic changes of cells took the same trend to reducing hormone levels arising from the effects of aflatoxin. Oestradiol showed positive correlation to histological changes for ovary and the uterus. Other workers have reported that oestradiol increases in plasma whenever aflatoxin consumption by animals increases (Amin *et al*., 2019). This disagreed with the current findings for

oestradiol, which concurred with the findings of Kourousekos *et al*. (2015), who reported increasing amount of oestradiolwith increasing quantities of aflatoxin.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The treatments with aflatoxin in the diets caused the reduction of the concentration of oestradiol, follicle stimulating hormone and luteinizing hormone in rabbit plasma compared to the control. Rabbit diets should not have more aflatoxin than the allowable concentrations of 50 ppb to avoid the reduction of hormones in plasma, whose effects may be observed through lowered rabbit fertility.

The different concentrations of aflatoxin intake by the rabbits in the current study had adverse histological effects on the hypothalamus, but did not affect the pituitary gland. The follicles within the ovaries showed necrotic outcomes among the treatments containing 200 and 400 ppb of aflatoxin. The study found reduced follicular number and sizes among the aflatoxin treated rabbits.

The different concentrations of aflatoxin in rabbit diets caused the reduction of the follicle sizes in the ovaries. The granulosa cells, zona pellucida and antrum of follicles in control treatment showed cuboidal morphological characteristics. The follicles in treatments with aflatoxin had columnar granulosa cell morphology.

Aflatoxin in the diets fed to female New Zealand white rabbits caused inflammatory changes to the uterus and vacuolization of the lamina propria of the fallopian tube. Effects of aflatoxin were clearly visible in the liver hepatocytes at treatments containing

aflatoxin levels above 100 ppb as observed through leukocyte infiltrations and the inflamed peripheral hepatocyte and oval cells. Rabbit diets should therefore contain aflatoxin of not more than the acceptable quantity of 50 ppb.

The study found positive and negative associations between the reproductive hormones under investigation in the current study and the histological changes in those organs that play significant roles in the reproductive processes of rabbits. The correlation between FSH and LH on one hand and the pituitary gland and also aflatoxin level were negative but that of aflatoxin and oestradiol was positive. The correlations between the liver and ovary necrotic changes against FSH and LH were positive but but FSH and LH gave negatively correlation to aflatoxin.

6.2 Recommendations

The diets of New Zealand white rabbits should not contain aflatoxin that exceeds the allowable quantity of 50 ppb because the lowest amount fed in the current study caused reduced concentrations of oestradiol, follicle stimulating hormone and luteinizing hormone in the blood plasma.

Aflatoxin in New Zealand white rabbit diets at concentration of 200 ppb caused inflammation and reduction of primordial follicles in the ovary and affected the neurosecretory cells of the hypothalamus. To save the follicles and the hypothalamus, the rabbit diets must always not be allowed to have aflatoxin in excess of the allowable concentration.

Aflatoxin should not exceed the acceptable levels of 50 ppb in rabbit diets because more of this will cause alterations in morphological characteristics like cuboidal or columnar granulosa cells of primordial, primary, secondary and tertiary follicles and these changes may result in their apoptosis.

Aflatoxin in the diets of New Zealand white rabbits at any concentration included in the current study caused inflammation of the liver hepatocyte and oval cells. This therefore necessitates the recommendation that the amount in the diet must not exceed the allowable quantities to save the liver, which is the prime target of toxins from any damage.

The negative correlations between the follicle stimulating hormone and luteinizing hormone, and aflatoxin imply that care must be exercised when feeding animals to ensure that aflatoxin is at least within allowable concentrations in the diet because excess will affect the rabbit's fertility

6.3 Further Work

There is need to design another study as a follow up to the current one to determine the effect of aflatoxin on ovarian weights. A second study should consider both the short and long term effects of aflatoxin on nutrient digestibility, intake and their availability for the function of oestrogen receptors in the liver, uterine epithelium and their effect on the overall fertility of female rabbits. Further work should also be carried out to establish the

cause of the reduction of FSH and LH in relation to the effect of aflatoxin on the pituitary gland. Its also recommended that further work be carried out to establish the effect of aflatoxin on female rabbit reproductive potency when both male and female are fed on different levels of aflatoxin laced diets.

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APPENDICES

Appendix I: Analyses of Variance

One-way ANOVA was carried out to determine whether the mean of the hormone levels differed significantly for the 12 rabbits which were assigned to the four treatment groups.

Hypothesis One

Ho1: There was no statistically significant difference between treatments for follicle stimulating hormone

H1: There was significant difference between treatments for follicle stimulating hormone

The findings (F (3, 44) = 0.978, p = 0.0412) revealed we fail to reject the null hypothesis since $p \leq 0.05$ and conclude that there was significant difference in FSH among treatment.

Appendix 1.1: ANOVA Findings

		Sum of Squares	df	Mean Square	F	Sig.
FSH	Treatments	1.074	3	.358	.278	.0412
	Error	16.107	44	.366		
	Total	17.181	47			

Oestradiol

Hypothesis Two

Ho1: There was no statistical difference between treatments for oestradiol hormone

H1: There was statistical difference between treatments for oestradiol hormone

The study results ($F (3, 44) = 6.269$, $p = 0.001$) indicated that we rejected the null hypothesis since $p < 0.05$. It was concluded that there was a statistically significant difference between treatments for oestradiol hormone.

Appendix 1.2: ANOVA Findings for Oestradiol

		Sum of Squares	df	Mean Square	F	Sig.
Oestradiol	Aflatoxin	859.285	3	286.428	6.269	.001
	Error	2010.221	44	45.687		
	Total	2869.506	47			

Luteinizing Hormone

Hypothesis Three

Ho1: There was no significant difference between treatments for luteinizing hormone concentration

H1: There was significant difference between treatments for luteinizing hormone concentration

The findings (F (3, 44) = 4.077, p = 0.012) indicated that we reject the null hypothesis since $p < 0.05$. This can be concluded that there was a statistically significant difference between treatments for luteinizing hormone.

Appendix1.3 : ANOVA Findings for luteinizing hormone

		Sum of Squares	df	Mean Square	F	Sig.
LH	Aflatoxin	0.088	3	.029	4.077	0.012
	Error	0.315	44	.007		
	Total	0.403	47			

Appendix II: Post-Hoc Test (Tukey)

The hormones (oestradiol, FSH and LH) that turned out significant which include in accordance with the ANOVA findings were subject to post hoc test for further analysis. The pairwise comparisons were conducted using the Tukey HSD test.

Oestradiol

The post-hoc analysis revealed that there was a statistically significant mean difference between the control treatment (1) and the treatment 4 (400 ppb of aflatoxin) ($p = 0.002$). The study also found a significant difference between treatment 2 (100 ppb of aflatoxin) and treatment 4 (400 ppb of aflatoxin) ($p = 0.013$).

Multiple Comparisons between Aflatoxin Treatment and Oestradiol

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control (Zero aflatoxin)	Diet containing 100 ppb of aflatoxin	1.84853	2.75943	0.908	-5.5192	9.2162
	Diet containing 200 ppb of aflatoxin	7.01435	2.75943	0.067	-.3533	14.3821
	Diet containing 400 ppb of aflatoxin	10.71509*	2.75943	0.002	3.3474	18.0828
Diet containing 100 ppb of aflatoxin	Control (Zero aflatoxin)	-1.84853	2.75943	0.908	-9.2162	5.5192
	Diet containing 200 ppb of aflatoxin	5.16583	2.75943	0.255	-2.2019	12.5335
	Diet containing 400 ppb of aflatoxin	8.86656*	2.75943	0.013	1.4989	16.2343
Diet containing 200 ppb of aflatoxin	Control (Zero aflatoxin)	-7.01435	2.75943	0.067	-14.3821	.3533
	Diet containing 100 ppb of aflatoxin	-5.16583	2.75943	0.255	-12.5335	2.2019
	Diet containing 400 ppb of aflatoxin	3.70073	2.75943	0.542	-3.6670	11.0684
Diet containing 400 ppb of aflatoxin	Control (Zero aflatoxin)	-10.71509*	2.75943	0.002	-18.0828	-3.3474
	Diet containing 100 ppb of aflatoxin	-8.86656*	2.75943	0.013	-16.2343	-1.4989
	Diet containing 200 ppb of aflatoxin	-3.70073	2.75943	0.542	-11.0684	3.6670

Luteinizing Hormone

The findings indicated that there was a statistically significant difference between the control treatment group and treatment 3 (200 ppb of aflatoxin) ($p = 0.043$). Significant difference was also found between treatment 1(control) and treatment 4 (400 ppb of aflatoxin) ($p = 0.013$).

Appendix : Multiple Comparisons between Treatment Means for LH

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	Upper Bound
Control (Zero aflatoxin)	Diet containing 100 ppb of aflatoxin	.08134	.03456	.102	-.0109	.1736
	Diet containing 200 ppb of aflatoxin	.09458*	.03456	.043	.0023	.1869
	Diet containing 400 ppb of aflatoxin	.11105*	.03456	.013	.0188	.2033
Diet containing 100 ppb of aflatoxin	Control (Zero aflatoxin)	-.08134	.03456	.102	-.1736	.0109
	Diet containing 200 ppb of aflatoxin	.01324	.03456	.981	-.0790	.1055
	Diet containing 400 ppb of aflatoxin	.02971	.03456	.825	-.0626	.1220
Diet containing 200 ppb of aflatoxin	Control (Zero aflatoxin)	-.09458*	.03456	.043	-.1869	-.0023
	Diet containing 100 ppb of aflatoxin	-.01324	.03456	.981	-.1055	.0790

Diet containing 400 ppb of aflatoxin	Diet containing 400 ppb of aflatoxin	.01647	.03456	.964	-.0758	.1088
	Control (Zero aflatoxin)	-.11105*	.03456	.013	-.2033	-.0188
	Diet containing 100 ppb of aflatoxin	-.02971	.03456	.825	-.1220	.0626
	Diet containing 200 ppb of aflatoxin	-.01647	.03456	.964	-.1088	.0758

Appendix III: The New Zealand white rabbits in the experimental unit before the start of the feeding trial (Source: Author, 2021)

Appendix IV: The feed requirements and schedule for rabbits in the tropics

The feed requirements for rabbits is determined by the following factors;

- The composition of the ration- a high energy feed will be taken in low quantity
- The environmental temperature
- The breed of rabbits being reared
- The size and age of the rabbits being fed
- The physiological status of the rabbit that is being fed

The young or sometimes orphaned kittens can be fed on liquid feed as follows;

Age (weeks)	Amount of milk(mls)	
1	6 - 7	Twice feeding daily
2	12 -13	"
3 - 6	15 - 25	"
6 - 8	30 + alfalfa+0.25 g of pellets or hay	"
8 days to 5 weeks	Pellets and hay	"

The feeding of growing rabbits to maturity takes place as follows;

Age(Weeks)	Live weight	Amount of feed(gm)	Supplements
6 - 12	2	100	Alfalfa
12 - 16	4	200	Alfalfa + hay
16 - 28	5	250	Hay
28 -42	6	400	Hay
Lactating Doe + Kittens for the whole lactation		250 Kg of pellets	Hay

Appendix V: The researcher returning the rabbits to the cage after weighing(Source: Author, 2021)

Appendix VI: The Phlebotomist bleeding the rabbits for hormonal assay from the medial ear vein(Source: Author, 2021)

Appendix VII: The Composition Of 10% Neutral Buffered Formalin

Component	Quantity
Sodium phosphate- monobasic	4.0 gm
Sodium phosphate- dibasic	6.5 gm
Formaldehyde 37%	100.0 ml
Distilled water	900.0 ml

Appendix VIII: The Rabbit attendant taking live Weights of rabbits(Source: Author, 2021)

Appendix IX: Humane sacrificing of Rabbits for scientific studies

Animal specie	Preferred Method	Alternative Methods
Rabbits	Pentobarbitone IP or IV	Inhahalation anaesthesia- overdose Stunning and exsanguination Cervical dislocation or decapitation Captive bolt penetration Over doss of anaesthetic – followed cervical dislocation or exsanguination

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