EFFECTS OF AFLATOXIN B_1 ON THE FUNCTIONING OF HYPOTHALAMO-PITUITARY-GONADAL (HPG) AXIS IN MALE PIGS (*Sus scrofa domesticus*)

BY

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NOVEMBER, 2017

DECLARATION

Declaration by Student

I declare that this study is my original work and has not been submitted to any university or institution of learning. All sources of information have been duly cited.

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DEDICATION

This work is dedicated to my dear parents, Benedict Mukumu and Maryanna Munini who inspired and encouraged me during my education. I also dedicate this thesis to my Missionary Benedictine family who supported me both financially and morally throughout my Master's education.

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God bless you all abundantly.

ABSTRACT

Aflatoxin is a major food contaminant, with adverse effects on the physiology of both humans and animals. Some pathophysiological effects of aflatoxicosis include reduction in growth rate, loss of weight, suppressed immunity, icterus, hemorrhagic enteritis, reduced performance, and consequently death. It has been reported that aflatoxicosis may interfere with reproduction by affecting animal fertility. This study investigated the effects of aflatoxin B_1 (AFB_1) on the functioning of the hypothalamo-pituitary-gonadal (HPG) axis in male pigs. The objectives of the study were: To investigate the effects of varied levels of AFB_1 on body weight and testicular volume; to determine the effects of varied AFB_1 levels on haematological indices; to determine the effects of different AFB_1 levels on follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone hormone (TH) and estradiol (E2); and to investigate the histopathological changes caused by AFB_1 on the testes, epididymis, liver, pituitary gland and the hypothalamus. Twelve reproductively mature male Large White pigs of the age of 7 to 9 months were randomly allocated to four groups; one control group and three treatment groups, each group comprising 3 pigs. They were housed in a pig pen. The AFB_1 doses were given in three levels. The first treatment group received 80 ppb per pig per day, second treatment group 160 ppb and the third treatment group 240 ppb per pig per day for 60 days. The control group received aflatoxin-free diet. Body weight and testicular volume were measured once per week. Blood samples were collected at an interval of 7 days for hemoanalysis and hormonal assays. The pigs were sacrificed immediately following termination of the treatment, and their tissues collected and processed for histological examination. Analysis of hormones was carried out using porcine ELISA kits for FSH, LH, testosterone and E2, in a sandwhich ELISA method. The study showed that AFB_1 levels had no significant effect on body weight (P=0.068) while testicular volumes significantly reduced (P=0.001) with increase in AFB_1 levels. White blood cells, red blood cells, haemoglobin and mean corpuscular haemoglobin concentration were significantly reduced (P=0.043, P=0.008, P=0.001, and P=0, respectively) in a dose-related manner. There was a significant increase in the levels of mean corpuscular volume (P=0.017) and mean corpuscular haemoglobin (P=0.047). AFB_1 had no significant effect on platelet count (P=0.086). Granulocytes were not significantly affected by the different aflatoxin levels (p=0.957), while lymphocytes and monocytes decreased significantly (p=0). The plasma levels of LH, testosterone and E2 of the AFB_1 challenged groups decreased significantly (P=0.042, P=0.002 and P=0.001, respectively) with increase in AFB_1 level; AFB_1 did not have significant effect on the level of FSH (P=0.197). Histopathological changes in the testes of the aflatoxin B_1-treated pigs were; progressive decrease of spermatogenesis cells, progressive reduction of Leydig cells and Sertoli cells, progressive increase in peritubular oedema, necrosis of seminiferous tubules and atrophy of the seminiferous tubules characterized by thickened basement membrane. Epididymis showed epithelia hyperplasia in all pigs treated with AFB_1. Histopathological changes in the liver tissue included; marked bile duct proliferation predominantly in the second (160 ppb) and third (240 ppb) treated groups; dilatation of the central vein; mononuclear cell infiltration; fatty change; fibrosis and marked congestion of the parenchyma. Histology sections of the pituitary gland and the hypothalamus showed normal structures. The findings of this study showed that oral administration of AFB_1 for an extended period of time induced marked physiological and histopathological changes in the testes, epididymis and the liver and may impair the functioning of hypothalamo-pituitary- gonadal axis in male pigs.

TABLE OF CONTENTS

DECLARATION ii
DEDICATION iii
ACKNOWLDEGEMENT iv
ABSTRACT v
TABLE OF CONTENTS vi
LIST OF TABLES x
LIST OF FIGURES xii
LIST OF PLATES xiii
LIST OF ABBREVIATIONS xiv
CHAPTER ONE 1
INTRODUCTION 1
1.1 Background to the Study 1
1.2 Statement of the Problem 4
1.3 Objectives of the Study 5
  1.3.1 General objective 5
  1.3.2 Specific objectives: 5
1.4 Research Questions 6
1.5 Justification of the Study 6
CHAPTER TWO 8
LITERATURE REVIEW 8
2.1 Hypothalamo-Pituitary-Gonadal Axis in Male Pigs (HPG Axis) 8
2.2 Role of the Liver in Hypothalamo-Pituitary-Gonadal Axis 9
2.3 Effects of Aflatoxin B_1 on Growth Rate 10
2.4 Effects of aflatoxin B_1 on Haematological Indices 11
2.5 Effects of Aflatoxins B_1 on Endocrine System and Reproductive System 12
  2.5.1 Effects of AFB_1 on endocrine system and reproductive system in animals 12
  2.5.1 Effects of AFB_1 on endocrine system and reproductive system in human beings 15
2.6 Histopathological Changes Caused by Aflatoxin B_1 17
CHAPTER THREE 21
MATERIALS AND METHODS 21
3.1 Animal selection 21

3.2 Sample Size and Power Calculation 21
3.2.1 Allocation of the animals to treatment groups 22
3.2.2 Animal care 23
3.2.3 Dose levels 23
3.3. Handling of Aflatoxin B_1 24
3.3.1 Preparation of Aflatoxin B_1 Solution 24
3.3.2 Administration of aflatoxin B_1 and feeds to the pigs 25
3.4 Determination of weight of pigs 26
3.4.1 Determination of testicular length, testicular width and scrotal circumference 26
3.4.2 Determination of testicular volume 27
3.5 Collection of Blood Samples for Haematology and Hormone Assays 27
3.6 Hormonal assays 28
3.6.1 Enzyme-linked Immunosorbent Assay (ELISA) test procedures for hormones 28
3.6.2 Step-wise procedure for the analysis of the hormones.................................29
3.6.2.1 Porcine Follicle-Stimulating Hormone (FSH)..29
3.6.2.2 Standard dilutions 29
3.7 Sacrificing the Animals..30
3.7.1 Extracting and processing of tissues 31
3.7.1.1 Dehydration 32
3.7.1.2 Clearing (de-alcoholization)...32
3.7.1.3 Infiltration (impregnation)...32
3.7.1.4 Embedding...32
3.7.1.5 Trimming..33
3.7.1.6 Dewaxing and staining 33
3.7.2 Photomicrography 34
3.8 Data Analysis and Presentation 34
CHAPTER FOUR...36
RESULTS 36
4.1. Effects of Aflatoxin B_1 on Body Weight and Testicular volume. 36
4.1.1 Effects of varied aflatoxin B_1 levels on body weight.....................................36
4.1.2 Effects of aflatoxin B_1 on testicular volume..37
4.2 Effects of Aflatoxin B_1 on Haematological Indices 39

4.2.1 Effects of AFB_1 on white blood cells (WBC's). 39
4.2.2 Effects of AFB_1 on red blood cells (RBCs) 40
4.2.3 Effects of AFB_1 on haemoglobin (Hb)....42
4.2.4 Effects of AFB_1 on mean corpuscular volume (MCV) 43
4.2.5 Effects of AFB_1 on mean corpuscular haemoglobin (MCH) 45
4.2.6 Effects of AFB_1 on mean corpuscular haemoglobin concentration (MCHC).46
4.2.7 Effects of AFB_1 on Platelets....47
4.2.8 Effects of AFB_1 on granulocytes....49
4.2.9 Effects of AFB_1 on lymphocytes....50
4.3 Effects of AFB_1 on Monocytes 50
4.4 Effects of Aflatoxin B_1 ON FSH, LH, TH AND E2 Hormone Levels 51
4.4.1 Porcine follicle stimulating hormone (FSH) 51
4.4.2 Porcine Luteinizing Hormone (LH) 53
4.4.3 Porcine Testosterone Hormone 54
4.4.4 Porcine Estradiol (E2) Hormone 56
4.5 Histopathological Changes Caused By Aflatoxin B_1....58
4.5.1 Histopathological changes in the testes....58
4.4.2 Histopathological changes in the epididymis induced by AFB_1 61
4.4.3 Histopathological changes in the liver induced by AFB_1 62
4.4.4 Histopathological changes in the hypothalamus and pituitary gland related to AFB_1 64
CHAPTER FIVE 66
DISCUSSION 66
5.1 Body Weight and Testicular Volume 66
5.2 Haematological Indices....66
5.3 Plasma Hormone Levels 68
5.4 Histopathological Changes Caused by aflatoxin B_1 69
CHAPTER SIX 73
CONCLUSION AND RECOMMENDATIONS....73
6.1 Conclusion 73
6.2 Recommendations....73
REFERENCES 74

APPENDICES 79
Appendix I: Definition of Operational Terms 79
Appendix II: Preparation of Aflatoxin B_1 Working Solution 80
Appendix III: Spectrophotometric Results for Follicle Stimulating Hormone 82
Appendix IV: Spectrophotometric Results for Luteinizing Hormone (LH) 83
Appendix V: Spectrophotometric Results for Testosterone Hormone 84
Appendix VI: Spectrophotometric Results for Estradiol (E2) 85
Appendix VII: Research Authorization 86
Appendix VIII: Research Permit 87

LIST OF TABLES

Table 4.1: Statistical difference in body weight between the groups using Fisher's least significant difference (LSD) 36
Table 4.2: Statistical difference in body weight within the groups 37
Table 4.3: Statistical difference in testicular volume in four groups using Fisher's least significant difference (LSD) 38
Table 4.4: Statistical mean difference in testicular volume within the groups 38
Table 4.5: Statistical difference in WBC’s in four groups using Fisher’s Least Significant Difference. 39
Table 4.6: Statistical mean difference in white blood cells (WBC’s) within the groups 40
Table 4.7: Statistical values obtained for RBCs in four groups using Fisher’s Least Significant Difference. 41
Table 4.8: Statistical mean difference of RBCs within the control and the treated groups 41
Table 4.9: Statistical difference of haemoglobin in four groups using Fisher’s Least Significant Difference 42
Table 4.10: Statistical mean difference of Hb within the control and within the AFB_1-treated groups 43
Table 4.11: Mean differences in MCV between the control group and the AFB_1 treated groups using Fisher’s Least Significant difference (LSD) 44
Table 4.12: Shows the mean difference in MCV within the four groups 44
Table 4.13: Mean differences in MCH between the control group and the AFB_1-treated groups using Fisher’s Least Significant difference (LSD) 45
Table 4.14: Shows the mean difference in MCH within the four groups 46
Table 4.15: Mean differences in MCHC between the control group and the treated groups using Fisher’s Least Significant difference (LSD) 46
Table 4.16: Statistical mean differences in MCHC within the control group and the experimental groups 47
Table 4.17: Statistical mean differences in platelet count between the control group and the experimental groups using Fisher’s Least Significant Difference (LSD) 48

Table 4.18: Statistical mean difference in platelet count within the control group and the experimental groups 48
Table 4.19: Statistical mean difference in granulocytes within the control group and the experimental groups 49
Table 4.20: Statistical mean differences in granulocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD) 49
Table 4.21: Statistical mean differences in lymphocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD) 50
Table 4.22: Statistical mean differences in monocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD) 51
Table 4.23: Statistical mean differences in the concentration of FSH between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference (LSD) 52
Table 4.24: Statistical difference in FSH concentration within the control group and within the AFB_1-treated groups 52
Table 4.25: Statistical mean differences in the concentration of LH between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference. 53
Table 4.26: Statistical mean differences in LH concentration within the control group and within the treated groups 54
Table 4.27: Statistical mean differences in the concentration of testosterone hormone between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference. 55
Table 4.28: Statistical mean differences in the concentration of testosterone within the control and the within the treated groups 56
Table 4.29: Statistical mean differences in the concentration of E2 between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference 57
Table 4.30: Statistical mean differences in the concentration of E2 within the control and the treated groups 58

LIST OF FIGURES

Figure 1: Chemical structures of the aflatoxin B_1, B_2, G_1 and G_2. Adopted from Pierre. (2012) 2

Figure 2. Diagrammatic representation of the main working components of the HPG axis. Retrieved from https://www.researchgate.net 9

LIST OF PLATES

Plate 1 to 11: Histopathological changes in the testes 60
Plate 12 to 15: Histopathological changes in the epididymis 61
Plate 16 to 27: Histopathological changes in the liver...64
Plate 28 to 35: Histopathological changes in the pituitary and hypothalamus 65

LIST OF ABBREVIATIONS

AF: Aflatoxin

E2: Estradiol

EDTA: Ethylenediamine tetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

FSH: Follicle stimulating hormone

GnRH: Gonadotropin releasing hormone

HPG axis: Hypothalamo-pituitary- gonadal axis

HRP: Horseradish Peroxidase

Kg: Kilogram

LH: Luteinizing hormone

MCH: Mean corpuscular heamoglobin

MCHC: Mean corpuscular haemoglobin concentration

MCV: Mean corpuscular volume

Ml: Milliliters

Ng/ml: Nanograms per milliliter

Nm: Nanometer

OD: Optical density

Pg/ml: Picograms per milliliter

Ppb: Parts per billion

RBCs: Red blood cells

RPM: Revolutions per minute

TH: Testosterone hormone

TMB: Tetramethylbenzidine

WBCs: White blood cells

WHO: World Health Organization

µl: Microliter

CHAPTER ONE

INTRODUCTION

1.1 Background to the Study

Aflatoxin (AF) is one of the contaminants in foods and feeds, with varying effects on the physiology of both animals and humans. It is a mycotoxin, a secondary metabolic by-product of the toxigenic fungi mainly *Aspergillus flavus* and *Aspergillus parasiticus* during their natural metabolic processes (Ahmed *et al.,* 2012). The four major groups of aflatoxin that have been described based on their fluorescence at chromatography are aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) (Dhanasekaran *et al*., 2011). The two main *Aspergillus* species yield different secondary metabolites. AFB_1 and B_2 are produced by *A. flavus* and *A. parasiticus* while AFG_1 and G_2 are produced by *A. parasiticus* (Ajani *et al*. (2014). In this classification, "B" and "G" stand for blue and green fluorescent colors respectively. The subscript numbers 1 and 2 indicate major and minor compounds, based on the degree of toxicity. AFB_1 is the most common, most toxic and a known carcinogen. It is the most widespread in the world (Bbosa *et al*., 2013).

Metabolic breakdown of aflatoxin B_1 and B_2 gives hydroxylated products designated M_1 and M_2 respectively (Dhanasekaran *et al*., 2011). These two are associated with milk and milk products from lactating animals once they ingest feeds contaminated with aflatoxin B_1 and B_2 respectively. The two metabolites, aflatoxin M_1 and M_2 remain stable during milk processing and can be readily moved down the food chain with adverse consequences on human beings (Bbosa *et al*., 2013).

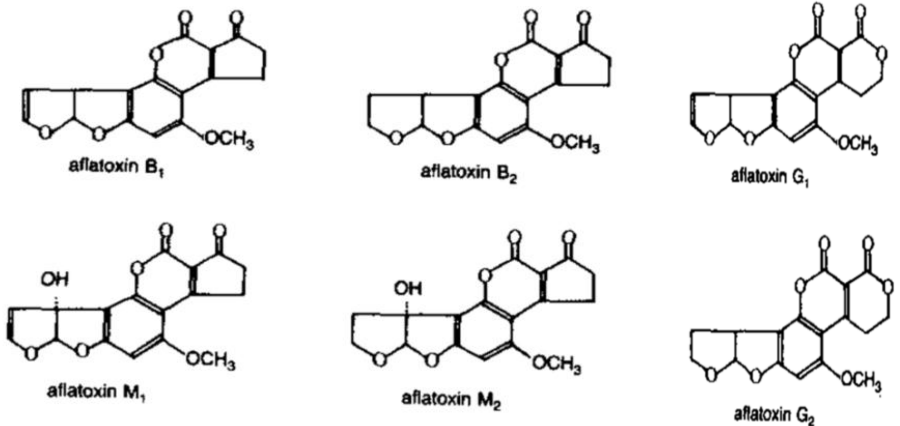


Figure 1: Chemical structures of the aflatoxin B_1, B_2, G_1 and G_2. Adopted from Pierre. (2012)

Aflatoxins are not transmissible between animals, thus, the main cause of the toxicity in humans and animals is consumption of aflatoxin contaminated food and feed stuff. The toxigenic fungi can contaminate various feed components like maize, rice, wheat, peanuts, millet and oily feedstuffs (Ajan *et al.,* 2014), some of which form the majority of common food for both humans and animals. This contamination is dependent on the prevailing environmental conditions such as temperature range of between 28 and 33^0C, relative humidity higher than 85% and oxygen availability, which favour production of aflatoxin in feeds (Bagheri *et al.,* 2014). Contamination of animal feeds with aflatoxin can occur during the growth of the crop while in the field, at harvest and during postharvest operations as well as in storage. Thus the task of preventing the occurrence of these toxins in animal feeds can be a very challenging one (Kanora & Maes, 2009).

According to Ajani *et al*. (2014), aflatoxins are highly lipophilic compounds which readily get absorbed through the gastrointestinal and respiratory tracts into the blood stream of animals and humans on exposure to the contaminants. The exposure to aflatoxins is through direct ingestion of aflatoxin-contaminated foods or through contaminated milk and milk products such as cheese and powdered milk, as well as

other animal tissues. Once ingested, these compounds diffuse across the cell membrane into the blood stream. They are then distributed to different body tissues and to the liver, which forms the main organ for aflatoxin metabolism (Bbosa *et al.*, 2013).

In lower experimental animals such as in male rats, aflatoxins have been shown to disrupt the reproductive system. Studies have shown that male rats fed on aflatoxin-contaminated feeds suffer pathological changes in the testicles and epididymis. These alterations in the testicles as well as the epididymis has related results in decreased number of spermatogenesis cells, spermatocytes as well as spermatids in these animals (Murad *et al.*, 2015). Such pathological changes in the reproductive organs, and in particular the testicles, may translate to reduced secretion of testosterone hormone, which is secreted by the testes in response to luteinizing hormone in males.

The effects of aflatoxin B_1 on the male reproductive system of lower animals has been shown to be duration dependent. Faridha *et al.* (2006) treated Swiss mice with aflatoxin B_1 to determine its toxic effects over different periods of time. Ninety-day old Swiss mice were divided into four experimental groups and given a daily dose of 50 µg/kg body weight for a period of 7 days, 15 days, 35 days and 45 days respectively. Histopathological analysis of the testicles and seminal vesicles of the mouse revealed a duration-dependent decrease in the weights of the testicles and seminal vesicles respectively. Generally, there was little impact of the treatment in the mice treated for duration of 7 days. On the other hand, mice treated for 15 days showed a significant decrease in the weight of testicles (73%), those treated for 35 days showed a decrease in the weight of testicles (68%) while those treated for 45 days showed a decrease in weight of testicles (51%). Weight of the seminal vesicles also decreased 76% in mice treated for 15 days, to 69% in those treated for 35 days and to 59% in mice treated for 45 days. These results revealed a duration-dependent regression of the testis and

seminal vesicles. Histological changes were also observed in both the spermatogenic and androgenic compartments of the testis. Leydig cells were shown to undergo hypertrophy and distortion of the nucleus shape following the treatment. These changes may also have an indirect effect on the secretion of testosterone hormone by the testes and secretion of 17ß-estradiol by the Leydig cells.

Aflatoxins cause adverse effects on poultry, young pigs, pregnant sows, calves and dogs. Osweiler (2014) gives tolerable dietary aflatoxin levels (in ppb) for different animal species whereby for young poultry is ≤50, adult poultry ≤ 100, weaner pigs ≤ 50, finishing pigs ≤ 200, dogs <50, calves <100 and in cattle <300 ppb. However, an animal's toxic response and disease are not only dependent on level of aflatoxin in feed, but also on species, sex, age, nutritional status and duration of intake (Hasanzadeh *et al*., 2011). This implies that prolonged feeding of low aflatoxin levels may results in reduced growth rate, immunosuppression among other clinical effects. In the liver (the major target organ), aflatoxins are metabolized into an epoxide that binds to macromolecules, especially nucleic acids and nucleoproteins (Osweiler, 2014) causing aflatoxin-DNA complexes and may cause hypoproteinemia leading to reduced growth rate. This study investigated the physiological effects of the aflatoxin B_1 on the functioning of the hypothalamo-pituitary-gonadal axis in male pigs.

1.2 Statement of the Problem

Exposure to aflatoxin B_1 has been known to pose a serious threat to both humans and animals particularly in the tropics, with varied pathophysiological effects. Studies on the chronic aflatoxicosis carried out in lower experimental animals reported that the effects of aflatoxins range widely from reduced growth, immunosuppression, decreased feed intake, carcinogenesis, mutagenesis, teratogenesis, as well as induced

hepatotoxicosis (Wild & Gong, 2009). However, the available information on chronic aflatoxicosis in pigs is inconsistent. According to Harvey *et al*. (1991), pigs are highly sensitive to the effects of aflatoxin (AF). Pigs get exposed to aflatoxin-contaminated diets due to the nature of feeding on foods or feed sources of unverified quality. They primarily utilize diets composed mainly of cereal grains, mostly wet feeds and research shows that toxic concentrations of aflatoxins are prevalent in grains such cereals as maize, rice, peanuts, wheat, cotton among others (Ajan *et al*., 2014), some of which form the majority of pig feeds. The current study focused on the effects of aflatoxin B_1 on the functioning of hypothalamo-pituitary-gonadal axis in male pigs, an area that has not been sufficiently investigated, in an attempt to establish the link between consumption of aflatoxin B_1-contaminated feeds and the risk of adverse functioning of the HPG axis in male pigs.

1.3 Objectives of the Study

1.3.1 General objective

To investigate the effects of aflatoxin B_1 on the functioning of hypothalamo-pituitary-gonadal (HPG) axis in male pigs

1.3.2 Specific objectives:

To investigate:

i) The effects of varied levels of AFB_1 on body weight and testicular volume.
ii) The changes in haematological indices of AFB_1 treated male pigs.
iii) The effects of different AFB_1 levels on follicle stimulating hormone, luteinizing hormone, testosterone and estradiol hormone concentrations.
iv) The histological changes caused by AFB_1 on the testes, epididymis, liver, pituitary gland and the hypothalamus.

1.4 Research Questions

1. How do varied aflatoxin B_1 levels affect body weight and testicular volume in male pigs?
2. What is the effect of varied aflatoxin B_1 levels on haematological indices in male pigs?
3. What is the effect of different aflatoxin B_1 levels on follicle stimulating hormone, luteinizing hormone, testosterone and estradiol hormone concentrations?
4. What is the effect of different aflatoxin B_1 levels on the histology of the testes, epididymis, liver, pituitary gland and the hypothalamus.

1.5 Justification of the Study

A number of studies have been done using different animal models such as birds, rats, mouse, calves and rabbits. However, the available information on the effects of aflatoxin B_1 on the physiology of pigs is inconsistent. In this study, pigs were selected as the model animals because pigs are often exposed to feeds of unspecified quality thus rendering them quite susceptible to AFB_1 contamination. This is because many farmers supplement pig feed from manufacturers with locally obtained feeds whose quality is untested. Although a lot has been done on the effects of aflatoxins in lower animals, physiologically a pig is very different from any other animal. In this study there was a possibility of getting different results from those realized from other model research animals. This because, although all mammals have common physiological processes, different animals have different ways of handling different challenges. This study therefore aimed at investigating the physiological and histological changes in pigs in response to varied levels of aflatoxin B_1. The HPG functional parameters investigated

on were the following: integrity of the hypothalamus, pituitary and gonads; synthesis, secretion and release of hormones so as to establish whether these parameters have an impact on the functioning of the HPG axis in male pigs. Body weight was also included as a part of the overall HPG axis functioning. This is because recent research has shown a close link between body mass index and semen parameters including sperm concentration or sperm count (MacDonald *et al*., 2013). High or low body weight was show to cause reduced semen quality as well as affect the concentration of reproductive hormones (Jensen *et al*., 2004). Thus body weight is a parameter closely linked to the functioning of the HPG axis, hence its inclusion in the present study.

CHAPTER TWO

LITERATURE REVIEW

2.1 Hypothalamo-Pituitary-Gonadal Axis in Male Pigs (HPG Axis)

The three cellular major components that comprise the HPG axis are; the Gonadotropin-releasing hormone (GnRH) neurons which project from the hypothalamus of the brain; gonadotropes in the anterior pituitary gland (adenohypophysis) which secrete the gonadotropins FSH and LH, and the somatic cells of the gonads; Leydig and Sertoli cells in the testis, theca and granulosa cells in the ovary (Tony, 2015). Gonadotropin-releasing hormone (GnRH) is secreted from the terminals of GnRH neurons in the hypothalamus (Crowley *et al*., 1985). GnRH is then released into the bloodstream which communicates the signal to gonadotrope cells in the adenohypophysis which has specific receptors for GnRH. Binding of GnRH to its receptors triggers the synthesis and secretion of FSH and LH which then travel via the systemic bloodstream to their target cells in the gonads (testis). FSH triggers secretion of inhibin B which has an inhibitory role on adenohypophysis in a negative feedback mechanism. LH triggers the synthesis and secretion of testosterone. Testosterone has a negative feedback effect on the hypothalamus and pituitary gland inhibiting release of GnRH, FSH and LH. Some effects of negative feedback triggered by testosterone occur via conversion to estradiol either in the testes or in the hypothalamus or pituitary gland. (Crowley *et al*., 1991) The basic system of the HPG axis is illustrated in figure 2.

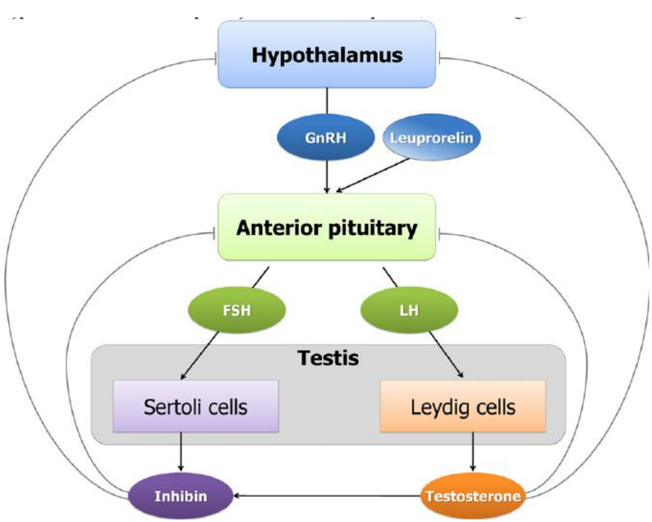


Figure 2. Diagrammatic representation of the main working components of the HPG axis. Retrieved from https://www.researchgate.net

2.2 Role of the Liver in Hypothalamo-Pituitary-Gonadal Axis

The liver forms the major site of synthesis and regulation of cholesterol and is responsible for 80% *denovo* synthesis. The liver plays a central role in the regulation of the levels of cholesterol in the body. It does this by synthesizing cholesterol which is exported to other cells, as well as by removing excess cholesterol from the body. This is done by converting any excess cholesterol into bile salts for excretion. The various lipoproteins involved in the transportation of cholesterol throughout the body are also synthesized by the liver (Vance & Vance, 2002). Cholesterol is the precursor of steroid hormones including sex steroids testosterone and estradiol (Hames & Hooper, 2005). Liver dysfunction has been shown to cause changes in serum levels of testosterone, free testosterone and sex hormone-binding globulin (Nitsche et al., 2014). Chronic liver disease has been shown to have clinical and biochemical evidence of hypogonadism in men associated with decreased testicular size, as well as of testicular atrophy in the histology of the testes. Liver disease has been shown to lower the levels of total testosterone. Thus, in addition to primary testicular failure, liver dysfunction has a

related hypothalamic-pituitary defect, although the nature and mechanism of the hypothalamic-pituitary defect remains unclear (Johnson, 1984).

The liver also forms the main organ for aflatoxin B_1 metabolism. It is in the liver where aflatoxin B_1 is oxidized by the enzyme cytochrome P450 to form a reactive epoxide which is responsible for a number of physiological effects observable at different body systems (McLean & Dutton, 1995). In the liver, the enzyme cytochrome P450 forms an intermediate through the addition of an oxygen onto the aflatoxin B_1 molecule, converting it to a highly reactive and mutagenic compound, AFB_1-8,9-epoxide (Turner *et al*., 2005). The primary role of the liver in aflatoxin B_1 metabolism makes it relevant in hypothalamo-pituitary-gonadal axis in this study.

2.3 Effects of Aflatoxin B_1 on Growth Rate

Effects of aflatoxin B_1 on growth rate is dependent on the AFB_1 concentrations and the duration of exposure. Feddern *et al*. (2013) highlighted the effects of varied aflatoxin B_1 concentrations in pig diets. Aflatoxin B_1 concentrations of 10-100 ppb in diet led to productivity losses with no noticeable clinical symptoms while AFB_1 levels of about 200-400 ppb brought about reduced growth and impaired feed efficiency in pigs. Levels of about 800-1200 ppb have been shown to impair growth of the animal, due to reduced feed intake.

An investigation of the ameliorative effect of vitamin E against the toxicity of aflatoxin B_1 on rats with special reference to its effect on male fertility was carried out by Elham & Mona (2004). The group that was treated with aflatoxin was given AFB_1 7.5 µg/200 grams body weight for a period of three successive weeks. The results showed a significant decrease in body weight and growth rate compared to the control group.

These results indicated a probable effect of aflatoxin B_1 on body weight and general growth in the animals.

2.4 Effects of aflatoxin B_1 on Haematological Indices

Aflatoxin B_1 has been shown to affect certain haematological parameters in different animals. Elham & Mona (2004) observed that AFB_1 significantly reduced red blood cell count, white blood cell count and haemoglobin concentration compared to that of the control in a group of rats given 7.5 µg/200 gram body weight of AFB_1 for three successive weeks. AFB_1 also significantly lowered PCV and MCHC than that of the control but significantly increased MCV and MCH, than that of the control. Abdel-Wahhab *et al*. (2002) had earlier observed that aflatoxin treatment significantly reduced blood haemoglobin, erythrocytes and leukocytes in male rats. Donmez *et al*. (2012) evaluated the toxic effects of aflatoxin B_1 on some haematological parameters in Merino rams and found out that aflatoxin B_1 caused decreased levels of erythrocyte, leukocyte count, hemoglobin concentration and hematocrit in rams fed 250 µg/day of aflatoxin B_1. The current study focused on male pigs which were treated with varying levels of aflatoxin concentrations to determine whether such doses had any effect on the haematologic parameters.

According to Bbosa *et al.* (2013), aflatoxin B_1 and its metabolites including the generated reactive oxygen species (ROS) have deleterious effects on the bone and blood cells. They also induce cancers on the hemopoietic system in bone marrow and lymphoid organs where blood, blood cells and blood components are produced. Blood system itself can be damaged by such toxins that affect production of blood cells, the components of blood including platelets, red blood cells and white blood cells, or the even affect the oxygen-carrying capacity of red blood cell or impair blood clotting.

Induced chronic aflatoxicosis has been shown to alter erythrocytes in rabbit (Verma & Raval, 1992). Such decrease in the number of circulating mature erythrocytes produce haemolytic anaemia.

2.5 Effects of Aflatoxins B_1 on Endocrine System and Reproductive System

2.5.1 Effects of AFB_1 on endocrine system and reproductive system in animals

Aflatoxins, particularly AFB_1, have been reported to impact on the endocrine glands and reproductive system at varying degrees, both in experimental animals and in humans. Hasanzadeh *et al*. (2011) did a study to determine the effects of aflatoxin B_1 on profiles of gonadotropic (FSH and LH), steroid (testosterone and 17β-estradiol) and prolactin hormones in adult male rat. The results showed that the level of FSH significantly increased in all treatment groups fed on aflatoxin B_1 contaminated food. The levels of luteinizing hormone and testosterone were lower in all of the treated groups while prolactin was significantly higher in the treated groups. The level of 17β-estradiol was significantly decreased only in the group that received higher concentration of aflatoxins in the diet. Their findings explain the hypophysotoxicity of aflatoxin B_1 and in particular on adenohypophysis. Effect of the toxin on the hypophysis could have led to the decreased level of the luteinizing hormone. The increased level of FSH in serum was attributed to the degeneration and desquamation of the epithelium, and a decrease in the size and thickness of the germinal layer in the seminiferous tubules. Damage to testes and a resulting reduction in circulating inhibin-B were suggested to elevate serum FSH levels. Serum testosterone levels were reduced mainly due to extreme damage to Leydig's cells. Oral administration of aflatoxin B_1 brought about tremendous increase in serum prolactin level in the study. The level of 17 ß-estradiol in the serum decreased only by administration of a relatively high dose of

aflatoxin B_1. Although the study explains the hypophysotoxicity of AFB_1, the results however, do not make any probable inference as to whether AFB_1 have any effect on the hypothalamic neurons responsible for the release of GnRH.

Aflatoxin B_1 can impact on the endocrine system as described by Bbosa *et al*. (2013). The effects of aflatoxin B_1 on the endocrine system are varied and are mainly as a result of disruption of enzymatic reactions in the various endocrine glands, which alter their normal functioning. Among the by-products of aflatoxin metabolism are the unstable reactive oxygen species (ROS), which cause abnormal cell proliferation resulting in cancers in different endocrine glands. This implies that the functions of the major endocrine glands including the hypothalamus and pituitary gland, and consequently the testes, are adversely affected by aflatoxin B_1 contamination (Bbosa *et al*., 2013). Cell tumours have also been observed in the ovary, adrenal glands, kidneys, thyroid gland, ovaries, testes, thyroid gland, parathyroid glands and endocrine pancreas (Bbosa *et al*., 2013). Consequently, the rate of hormone secretion by the different endocrine glands is altered. The plasma testosterone and luteinizing hormone (LH) concentrations have been reported to reduce in aflatoxin-fed birds (Lakkawar *et al*., 2004). Related changes are also expected on the physiology of other body systems since the body of an individual functions as a unit. The current study investigated the effects of aflatoxin B_1 on hypothalamus, the pituitary gland and the gonads as a single entity, so as to give whole picture of how toxic levels of aflatoxin B_1 affect the entire reproductive hormonal axis in male pigs.

Testosterone concentration and sperm production were determined in West African dwarf bucks fed varied levels of dietary aflatoxin B_1 (Ewuola *et al*., 2014). They subjected the bucks to varied dietary AFB_1 for a period of 12 weeks so as to determine their reproductive potentials. The goats in the control group were fed diets with 0 µg/kg

while the test groups were given 50 μg/kg, 100 μg/kg and 150 μg/kg respectively. The gonadal sperm reserves, estimated as the total number of late spermatids and spermatozoa in the testicular tissues and the extra-gonadal sperm reserve, estimated as the total number of spermatozoa in all the sections of epididymal tissues, were significantly higher in the control group compared to the test groups. The significant decrease in the gonadal sperm reserves with increased AFB_1 concentration was attributed to the degeneration of sertoli cells due to aflatoxin B_1 in the diet. Goats fed higher aflatoxin levels had significantly lower testosterone levels, which could be attributed to impairment of Leydig cells by the increased toxic levels. This may also explain the reduced spermatozoa production in the testis. Another significant observation was the decline in daily sperm production with increased aflatoxins in the diet, which can be attributed to impaired Leydig cells and Sertoli cell degeneration (Ewuola *et al*., 2014). Testicular glucose and total protein concentrations were greatly reduced, probably as a result of impaired utilization and metabolism of these substrates due to aflatoxin contamination. This might have an indirect impact on testicular parameters and overall growth rate of the animal.

Chronic male reproductive toxicity was also investigated in Swiss mouse and Wistar rat by Akbarsha *et al*. (2008). They treated the rats with aflatoxin B_1 at a concentration of 20 μg per kg body weight per day via intra-peritoneal route. The investigations demonstrated that aflatoxin B_1 is severely toxic to male reproductive mechanisms in mouse and rats. AFB_1 induced severe histopathological changes in the testis, affecting both spermatogenic and androgenic areas of the male reproductive system. The seminiferous epithelium was severely disrupted hampering both mitotic and meiotic division of the germ cells. The affected germ cells are prematurely released from the sertoli cells. The consequent effects of AFB_1 contamination in this case was impaired

spermatogenesis, decreased sperm counts, impaired motility and viability of spermatozoa. Aflatoxin B_1 was also shown to have carcinogenic potential on the epididymis. Thus, chronic exposure of humans and animals to aflatoxins B_1 which is possible through dietary contamination, particularly in the tropical climate of developing countries, can bring about deterioration of male reproductive health and fertility can be compromised as a result (Akbarsha *et al*., 2008).

Ovarian follicular growth and atresia were observed in rats fed different concentration of AFB_1 (Hasanzadeh & Amani., 2013). An increased concentration of aflatoxin B_1 in the body causes increased follicular destruction. AFB_1 causes follicular atresia, which reduces the follicular pool in the ovary. The number of quiescent as well as developing follicles is significantly reduced and the resulting effect may lead to either permanent or explicit infertility or sterility in the rat. This implies that AFB_1 contamination can have adverse effects on the reproductive potential of female rats by causing developmental defects on the ovarian follicles as described by Hasanzadeh & Amani (2013).

2.5.1 Effects of AFB_1 on endocrine system and reproductive system in human beings

A number of studies have also been done on humans to assess whether aflatoxin B_1 contamination has an impact on the endocrine and reproductive functions in both males and females. Mohammed *et al.* (2014) conducted a study to determine aflatoxins in semen of men with unexplained infertility and to establish the relationship between aflatoxin and semen parameters. The results of this study showed presence of aflatoxin B_1 (AFB_1) in 25% of the semen of infertile patients, compared to 2.1% among the controls (P=0.0082). Another significant finding in this study was abnormal semen

parameters that included, severe reduction in sperm count, reduced motility, high percentage of abnormal morphology, and high viscosity in the semen of the infertile group compared to the fertile group and the WHO reference values for normal semen parameters (Cooper, 2009). Although there are several factors that may contribute to infertility in humans, including an increase in contraception, decline in semen quality contributes significantly to male infertility. Environmental agents, such as molds have been considered to play an important role in the decline of semen quality. The presence of aflatoxin in the semen of infertile men may be used to explain the observable abnormal semen parameters in this study group. The main cause of aflatoxin contamination for the studied group is likely to be nuts and seeds, herbs and medicinal plants, spices, dried vegetables and cereal grains which form the reliable meal for the group studied. Contamination of human food with aflatoxin B_1 has been known as a major cause of delayed testicular development and testicular degeneration. These effects are consequently related to reduced reproductive potential in such men. The testis undergoes morphological regressive changes while the Leydig cells are seriously impaired (Mohammed *et al*., 2014). Another remarkable finding is formation of aflatoxin-DNA complexes, referred to as DNA-adducts. These complexes are formed when aflatoxin couples with DNA in the cell nuclei. This complex might help to explain the abnormalities in the human sperm.

Similar investigations were carried out in females by El-Azab *et al*. (2010). This was to determine the presence of aflatoxins in the blood of infertile females in Egypt, as well as to ascertain whether there is any relationship between the presence of aflatoxins and alterations in human ovulatory functions and hormonal levels. Aflatoxin B_1 analysis yielded negative results. However, there was a significant enlargement in the mean ovarian volume but a significant decrease in follicular size. Their results also

showed significantly higher levels of luteinizing hormone and significantly lower levels of mid-luteal progesterone in infertile females. The findings of this study were quite different from other related studies regarding hormonal levels.

2.6 Histopathological Changes Caused by Aflatoxin B_1

Aflatoxins have been shown to cause histopathological changes in the liver. Kettere *et al*. (1982) reported on acute aflatoxicosis in pigs fed mouldy peanut containing 22,000 µg aflatoxin B_1 per Kg. The clinical signs included extensive centrilobular liver necrosis and haemorrhage, hepatic centrilobular cellular infiltration, hepatocyte swelling, hepatocyte vacuolation together with bile stasis and bile ductule hyperplasia. The acute toxicity caused death in pigs within twelve hours.

In lower experimental animals, AFB_1 has been shown to induce severe histopathological changes in the testis, epididymis and endocrine glands in animals in a dose-dependent manner (Bbosa *et al.*, 2013). Murad *et al.* (2015) carried a study on the toxic effect of aflatoxin B_1 on reproductive system of albino male rats. Results from this study showed adverse effects of aflatoxin B_1 on the reproductive system of male rats. These effects included observable pathological changes in testicles and epididymis, with degeneration and necrosis of epithelial cells of sperm tubules and reduced number of sperms inside the cavity of the tubules. The number of Leydig cells and the height of seminiferous tubules in animals treated with AFB_1 were significantly reduced compared to the control group. The number and index of Sertoli cells, including the number of spermatogenesis cells, spermatocytes as well as spermatids was significantly reduced with increased toxin concentration. The results from this study indicated that aflatoxin B_1 causes notable pathological changes in the testicles and epididymis in Albino male rats.

Pathological changes have also been observed in the testes and epididymis as reported by Ortatatli *et al*. (2002). They conducted a study to determine the pathological changes in testes and epididymis and plasma testosterone levels in adult roosters treated with different levels of total aflatoxin (AF: B_1, B_2, G_1, G_2) for 8 weeks. Gross examination showed that the testes of all aflatoxin treated groups were significantly atrophied compared to the control group. Histopathological changes included absence of spermatogenesis cells in the testes of the aflatoxin treated groups. Abnormal spermatozoa, mononuclear cell infiltration and/ or focal lymphoid cell accumulation in the intertubular areas of the testes and epididymis were observed in all the aflatoxin treated roosters. Other observations included degeneration and desquamation in the epithelium and decrease in the size and thickness of the germinative layer of the seminiferous tubules as well as lowered plasma testosterone levels in the adult roosters treated with aflatoxin. This study investigated if similar histopathological changes occur in the testes and epididymis of pigs treated with different levels of AFB_1 only for the same duration of time.

Histomorphological study was carried out in rats by Hasanzadeh & Rezazadeh, (2013). They fed the rats different aflatoxin levels orally. Oral administration of the treatment was considered to be consistent with normal toxin intake thus producing both relevant and reliable results. Aflatoxin B_1 resulted in disrupted integrity of the epithelia of seminiferous tubules. The population of spermatids and spermatozoa was greatly reduced. Lakkawa *et al*. (2004) observed similar testicular changes in an earlier experiment on aflatoxin B_1 toxicosis in young rabbits. The reproductive organs of the treated group showed regressive changes of varying degree on the germinal epithelium of the seminiferous tubules. Consequently, severe dystrophic alteration of the spermatogenic epithelium including oedematous interstitial tissue occurred. The testes

of the treated group also showed varying degrees of dystrophy, a factor that might have contributed to atrophy of spermiogenic epithelium and to tissue oedema. Such adverse effects of aflatoxin B_1 on the male reproductive organ are responsible for reduced number of mature spermatozoa and increased number of abnormal or dead sperms observed in the treated group. These results show that aflatoxin contamination impairs the reproductive potential in rats and rabbits.

Histopathological investigation was carried out on rats by Elham & Mona (2004). They fed the rats 7.5 mg/200 grams body weight of aflatoxin for 3 weeks. The liver in this experiment showed hydropic and vacuolar degeneration of hepatocytes, mainly around the congested blood vessels. Other observations were lymphocytic cellular aggregation. Inflammatory cellular infiltration of portal area mostly lymphocytes and congestion of portal vessels with presence of small newly formed bile ducts were noted. The testicles of this group showed degenerated lining of epithelium of seminiferous tubules and congestion of testicular blood vessels with intertubular oedema. Coagulative necrosis of entire lining epithelium of some seminiferous tubules was also noted. Microscopic examination of the brain tissue of rats in this group showed meningitis represented by congestion of menigeal blood vessels with inflammatory cellular infiltration mostly lymphocytes and few neutrophils.

Similar results were described by Bbosa *et al.* (2013) where they report aflatoxin B_1 causes pallor discoloration and enlargement of liver, congestion of liver parenchyma, cytoplasmic vacuolation or fatty change of hepatocytes, necrosis of hepatocytes and newly formed bile ducts, mononuclear and heterophilic cell infiltration in broiler chicks. Marai & Asker (2008) while reporting on histopathological effects of aflatoxin highlighted that administration of daily AF doses as low as 0.04 mg/kg body weight resulted in bile duct proliferation, perivascular oedema, fibroblastic infiltration, dilated

lymphatic ducts and loss of glycogen in the liver tissue of calves. Although gonadal changes in different animals have been documented, pigs, with their unique physiology are expected to give different results. This study therefore focused on the effects of AFB_1 on hypothalamo-pituitary-gonadal axis in reproductively mature male pigs to determine if varied AFB_1 levels cause any related histological changes in male pigs.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animal selection

This research adopted an experimental design using twelve reproductively mature large white pigs of the age of 7 to 9 months and of initial average body weight of 54 kg. This is because the large whites are widely available and adaptable. The pigs were obtained from the University of Nairobi and housed in a pig pen at Karen in Nairobi. Purposeful consideration for inclusion and exclusion from the study group was based on the gender, breed, age and health status of the animal. The pigs were allowed one week acclimatization period during which they were subjected to general medical screening. The pigs were dewormed with Doramectine 10mg/ml to eradicate any internal and external parasites. The animals were randomly allocated into four groups, comprising of one control and three treatment groups, each group comprising 3 pigs. This number was considered sufficient per study group since each animal is a physiological unit of its own.

3.2 Sample Size and Power Calculation

A minimum number consistent with achieving the scientific objectives for this study was used. To achieve a statistical power of 80% (β=0.80) and a critical limit of 95% (α=0.05) for significance, a minimum of 12 animals was sufficient for this study. The sample size was calculated using the statistical formula below, as proposed by Dell *et al*. (2002):

$$n = C \times (p_c q_c + p_e q_e) / d^2 + 2 / d + 2$$

Where:

P_c = The estimates for proportion of the control group exhibiting the event

P_e = The desired proportion of the experimental groups exhibiting the event.

$q_c = 1 - p_c$; $q_e = 1 - p_e$; and d is the difference between P_c and P_e, expressed as a positive quantity.

C is a constant whose value is 7.85 for $\alpha = 0.05$ and $1-\beta = 0.8$

In this study, P_c of 0.2 and P_e of 0.8 were estimated for the control and experimental group respectively.

Sample size was calculated as:

$n = 7.85 \times (0.2 \times 0.8)/0.6^2 + 2/0.6 + 2$

n= 12.31

Twelve animals were randomly chosen for this study.

3.2.1 Allocation of the animals to treatment groups

This study adopted a method that was used by Ewuola *et al*. (2014). Experimental research approach, with one control group and three treated groups was used. Animals in each study group were marked by individual ear tags.

The animals were tagged as follows:

1st treated group: AI, BI and CI respectively.

2nd treated group: AII, BII, and CII respectively.

3rd treated group: AIII, BIII and CIII respectively.

Control group were marked as Ac, Bc, and Cc respectively.

3.2.2 Animal care

The pigs were housed in concrete floor pens which were kept dry with dry straw, except for the dunging area. The straw was changed after every three days. Cleaning for the dunging area was done daily to remove the pig's wastes and reduce odour, while the sleeping area and the free space for movement were cleaned after every three days. The entire space in each of the rooms was 64 square feet. Each room housed three pigs. These rooms were further partitioned with hardwood to allow each of the three pigs its own feeding and sleeping area. The partitions were firmly fixed to support the weight of a pig when rubbing itself against the panes or rooting at the base of the panels. The conditions of the pens were maintained similar in terms of space, bedding, cleaning, feeding and water supply. The housing conditions were maintained at 12:12 dark: light cycle. The prevailing environmental temperature at the study area was within the range of 12.5 ^{0}C to 26.8^{0}C, while relative humidity ranged between 55% and 71%. House ventilation was adequate. Measurements for the different parameters were done at consistent time intervals.

3.2.3 Dose levels

The pigs in the treatment groups were subjected to three levels of aflatoxin B_1 administered orally. Animals in the 1st treatment group were given 80 ppb of AFB_1 per pig per day, 2nd treatment group 160 ppb AFB_1 per pig per day and the 3rd treatment group 240 ppb AFB_1 per pig per day for a period of 60 days as per Ahmed *et al*. (2012) method modified.

3.3. Handling of Aflatoxin B_1

Standard operating procedures for aflatoxin B_1 were carried out as provided by United States Department of Agriculture (USDA) Aflatoxin Handbook (2002). Protective clothing and nitrile gloves were worn at all times. Standards were prepared in a fume cupboard while wearing a mask. Aflatoxin B_1 is sensitive to light and therefore was stored in brown vials that were wrapped with aluminum foil protected from sunlight and stored in a fridge (Samsung refrigerators “built for Africa”, Germany) at temperature 2-8^{0}C. Used glassware such as funnels and beakers were completely submerged in 5% (v/v) Sodium hypochlorite and soaked for at least 5 minutes for disinfection. The items were removed from the bleach solution, submerged in a dish washing liquid/ water solution, washed thoroughly, then rinsed with clean water before reusing. Disposable materials such as cuvettes vials, eppendorfs and pipette tips were soaked in Sodium hypochlorite for at least 5 minutes. After pouring off the disinfectant down the drain the materials were placed in a garbage bag and discarded.

3.3.1 Preparation of Aflatoxin B_1 Solution

A standard amount of aflatoxin B_1 of ninenty milligrams was purchased from Bora Biotechnology in Nairobi- Kenya. This was then prepared into solution form as per the manufacturer’s instructions. Preparation of the stock solution of aflatoxin B_1, as well as further dilutions into the specified quantities for each animal was done in analytical science laboratory at Masinde Muliro University of Science and Technology. The materials required were aflatoxin B_1, analytical balance, aluminium foil, distilled water, methanol, dichloromethane, nitrile gloves, cotton wool, 70 % ethanol, blue micropipette tips, yellow micropipette tips to allow change of tips between samples, micropipettes (1000 µl 200 µl and 5µl), dispensing bottles, mouth masks and a

permanent marker. Aflatoxin B_1 dosage was prepared in the ratio of 1:2:3 with 80 ppb for the 1st treatment group, 160 ppb for the 2nd treatment group and the 240 ppb for the 3rd treatment group.

In preparation of aflatoxin B1 stock solution, 84.6 mg of aflatoxin B1 was weighed on KERN ABS 80-4N analytical balance and then transferred to a conical flask. The aflatoxin B1 was dissolved in 8.64 ml of dichloromethane and methanol in a ratio of 1:1 to give a clear colourless stock solution of the aflatoxin B1 concentration of 10 mg/ml in dichloromethane and methanol; therefore 8.64 ml of dichloromethane/methanol was used to dissolve the AFB1. This means that 4.32 ml of dichloromethane and 4.32 ml of methanol was used to give the ratio of 1:1. Administration of AFB1 was done in 3 doses, which are 80 ppb, 160 ppb and 240 ppb. Each dosage was topped up with distilled water to make a final volume of 75 ml of aflatoxin B1 in each dispensing bottle. (Appendix II).

3.3.2 Administration of aflatoxin B_1 and feeds to the pigs

Oral route for administration of aflatoxins B_1 was used, whereby the predetermined aflatoxin B_1 concentrations were mixed with 150g of pig finisher's feed and served to each pig in separate aluminium feeding pot, each in their specific experimental group. This mode of administration was preferred because the oral route is considered to be consistent with the normal feed intake in pigs, making the results relevant and reliable (Hasanzadeh & Rezazadeh, 2013). The first treated group received AFB_1 dosage of 80 ppb, the 2nd treatment group 160 ppb and the 3rd treatment group received 240 ppb aflatoxin B_1 dosage in their daily feeds for 60 days. Following the administration of the treatment to the experimental group, each pig was served its daily 2kg feed in a separate aluminium feeding pot. This made it possible to quantify and monitor the amount of

feed for each pig. The control group was fed aflatoxin-free diet. Pig-finishers feed was obtained from Unga feeds Company in Nairobi. The distribution of the feed was done every morning at 8.00 A.M. This is because pigs are quite sensitive to changes in routine. Water was provided *ad libitum*. The experimental group was observed twice daily and weighed once each week for 60 days. Individual feed consumption was also monitored and recorded. Baseline data regarding body weight, testicular length, testicular width and scrotal circumference as well as blood sampling was done on the first day of study, prior to the administration of aflatoxin B_1 to the experimental groups.

3.4 Determination of weight of pigs

The weight of pigs in this study was determined as per the method described by Groesbeck *et al*. (2002). A commercial weigh band was used in determining the weight of each individual pig. The procedure involved measuring the girth of the chest immediately behind the elbow of the pig, then reading the weight in kilograms as indicated on the band. Three measurements were taken for each pig every time weight was being determined, and their average obtained to minimize errors. Weight measurements for each pig were taken on day zero, then weekly throughout the entire period of study and recorded for analysis.

3.4.1 Determination of testicular length, testicular width and scrotal circumference

Gonadal changes including testicular length, testicular width and scrotal circumference were determined weekly using an ordinary measuring tape in centimeters. The recorded number of observations for each of the parameters was 96 measurements, 8 measurements per pig. Observations were carried out twice each day to monitor any behavioral changes or any occurring sign of toxicity, and recorded.

3.4.2 Determination of testicular volume

Testicular volume was determined using a formula proposed by Lin *et al.* (2009) for measuring testicular dimensions.

Volume = Length x $(\text{Width})^2$ x 0.59

$V = L \times W^2 \times 0.59$

3.5 Collection of Blood Samples for Haematology and Hormone Assays

Five milliliters of blood sample was collected from the pigs' ear once every week in vacutainer tubes containing Ethylenediamine tetraacetic acid (EDTA) as an anticoagulant. The blood was collected from either the right or left ear of each of the pigs using vacutainer needles G21 and vacutainer needle holders. The blood samples were analyzed for haematological indices immediately after collection. This was done using an automatic haemoanalyzer (Model PCE-210N Japan) at St. Odilia's dispensary in Nairobi. The haematologic indices investigated were the total white blood cell count (WBCs), total red blood cell count (RBCs), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) and platelets. A total of 120 blood samples, 10 for each pig, were subjected to haemoanalysis. Plasma samples were prepared by incubating the whole blood at room temperature for 10-20 minutes. The tubes were centrifugated for 20 minutes at 2,000-3,000 rpm. The supernatant was carefully collected and stored in a freezer (Samsung refrigerators "built for Africa", Germany) at -21^0C for hormonal analysis.

3.6 Hormonal assays

A total of 84 plasma samples, 7 from each pig, were subjected to hormone assays at University of Nairobi, Kabete Campus, Department of Clinical Studies. The apparatus and reagents for the procedure included the following: Microplate reader (MR-96A Mindray microplate reader), micropipette (humapette), NaOCl 3.5 % m/v for disinfection, multichannel pipette, ELISA kits obtained from *Invitro Diagnostics limited* - Nairobi and the accompanying supplies for the analysis.

3.6.1 Enzyme-linked Immunosorbent Assay (ELISA) test procedures for hormones

ELISA kits were employed for hormonal assays in quantitative determination of FSH, LH, Testosterone and estradiol in plasma samples for each individual pig following blood sampling for the control group, and the three treated groups. Sandwich ELISA was carried out for each of the hormones using the following kits: Porcine follicle-stimulating hormone ELISA Kit for FSH, porcine luteinizing hormone (LH) ELISA kit, porcine testosterone ELISA kit and porcine estradiol ELISA kits. Sandwich-ELISA method was used in the analysis of all these hormones. The microelisa stripplate provided in the test kits had been pre-coated with an antibody specific to each hormone. Standards or samples were added to the appropriate microelisa stripplate wells and combined to the specific antibody. A Horseradish Peroxidase (HRP) - conjugated antibody specific for each hormone was added to each microelisa stripplate well and incubated. Free components were washed away. The TMB substrate solution was added to each well. Only those wells that contain the specific hormone and HRP conjugated hormone antibody appeared blue in color and then turned yellow after the addition of the stop solution. The optical density (OD) was measured spectrophotometrically at a

wavelength of 450 nm. The OD value is proportional to the concentration of each hormone in the sample. The concentration of the FSH, LH, testosterone and estradiol in the samples were calculated by comparing the optical density of the samples to that of the standards in a standard curve. The mean OD values for each group was then calculated, and the concentration of each hormone determined using specific concentration curves.

3.6.2 Step-wise procedure for the analysis of the hormones

3.6.2.1 Porcine Follicle-Stimulating Hormone (FSH)

The procedure for determining the concentration of FSH, LH, testosterone and estradiol in the sample was as provide in each test kit manual (*Microwell Elisa diagnostic systems, United States of America).*

3.6.2.2 Standard dilutions

Ten wells were set for standards in a microelisa stripplate. In well 1 and well 2, 100µl standard solution and 50 µl standard dilution buffer were added and mixed well. In well 3 and well 4, 100µl solution from well 1 and well 2 were added respectively. Then 50µl standard dilution buffer were added and mixed well. 50µl solution was discarded from well 3 and well 4. In well 5 and well 6, 50µl solution from well 3 and well 4 were added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 7 and well 8, 50µl solution from well 5 and well 6 were added respectively. Then 50µl standard dilution buffer were added and mixed well. In well 9 and well 10, 50µl solution from well 7 and well 8 were added respectively. Then 50µl standard dilution buffer were added and mixed well. 50µl solution was discarded from well 9 and well 10. After

dilution, the total volume in all the wells were 50µl and the concentrations were 36 ng/ml, 24 ng/ml, 12 ng/ml, 6 ng/ml and 3 ng/ml, respectively.

In the microelisa stripplate, one empty well served as a blank control. In sample wells, 40µl sample dilution buffer and 10µl sample were added (dilution factor is 5). Samples were loaded onto the bottom without touching the well wall, then mixed well with gentle shaking. The sample was kept in a fridge (LG electronics, U.S.A) at 37^{0}C for 30 minutes after sealing with a closure plate membrane. The concentrated washing buffer was diluted with distilled water 30 times. After peeling off the closure plate membrane, the wells were refilled with wash solution and allowed to rest for 30 seconds. The wash solution was discarded. The washing procedure was repeated for 5 times to remove unbound materials. 50µl HRP- conjugate reagent was added to each well except the blank control well. Washing was done as earlier described. 50µl chromogen solution A and 50µl chromogen solution B was added to each well, mixed by gentle shaking and incubated at 37^{0}c for 15 minutes in the absence of light. 50µl stop solution was added to each well to terminate the reaction. The color in the well changed from blue to yellow. Absorbance O.D. was read at 450nm using a microtiter plate reader (MR-96A Mindray microplate reader model, Germany) and the results printed on a sheet of paper. Similar procedure was followed for the determination of the porcine luteinizing hormone, testosterone and estradiol hormone concentrations in the sample.

3.7 Sacrificing the Animals

Treatment of the animals with AFB_1 was terminated one day before sacrificing the animals. Thus, the entire experimental period lasted 60 days of administering AFB_1 to the treatment groups. Following the termination of the treatment, the pigs were sacrificed using a captive bolt pistol which was used to render the animal unconscious.

When the animal was fully stunned, the throat was cut with a sharp knife and allowed to bleed. Horizontal bleeding of the carcass was carried out on a clean table, allowing each carcass sufficient space and time for the carcass to drain blood until only negligible flow could be noticed.

3.7.1 Extracting and processing of tissues

The liver, hypothalamus, pituitary gland, testes and epididymis were collected immediately after sacrificing the animal to prevent postmortem autolysis and decomposition. A longitudinal craniotomy was carried out using hacksaw blade and forceps in the collection of the pituitary and hypothalamus. The skull was split ventrally and dorsally along its axis to expose the brain tissue, taking caution not to damage the brain and then the pituitary and the hypothalamus were sectioned out from the intact brain tissue for processing. A total of 60 tissues were collected, five from each pig. The blocks of tissues measuring 5mm thick were prepared. These were put in labeled sample containers and immersed completely in 10% formalin for fixation. The tissues were fixed for duration of 72 hours. The blocks of tissues were then forwarded to Moi teaching and referral hospital, histopathology department for processing and examination. The tissue processing procedure involved the following seven steps: Dehydration, clearing, infiltration/impregnation, embedding, sectioning, floating of sections and mounting on glass slides, dewaxing and staining of the sections for microscopy.

3.7.1.1 Dehydration

The dehydrating agent used was ethanol. The specimens were immersed in a series of ethanol solutions from as follows: 70% ethanol for 30 minutes, 90% ethanol for 30 minutes, and then absolute ethanol for 30 minutes (x3). Water was gradually removed from the tissues thus avoiding shrinkage of the cells and the alternation of the tissues.

3.7.1.2 Clearing (de-alcoholization)

The clearing agent used was Xylene. This was done done in a multiple of changes as follows: Xylene/ethanol (1:1) for 20 minutes (x2), then 100% Xylene for 30 minutes three times. The aim was to completely displace ethanol from the tissues.

3.7.1.3 Infiltration (impregnation)

Molten paraplast was infiltrated into the tissues to displace ethanol (the clearing agent) and to provide internal support to the tissues during sectioning. The molten paraplast used for infiltration was kept in an electrically heated, thermostatically controlled oven (J P Selecta S. No. 324596, Spain) at 58^{0}C, slightly higher than the melting point of paraplast which is 56^{0}C so as to ensure that the paraplast remained molten at all times. Infiltration was done in a series of steps as follows: The tissues were transferred into a mixture of xylene saturated with paraplast in a ratio of 1:1. These were placed on top of the paraffin oven, in some warm spot at 35^{0}C, for 15 minutes. The tissues were then placed into the molten paraplast for 30 minutes in two changes, then for 1 hour.

3.7.1.4 Embedding

The infiltrated tissues were placed in an embedding mold and cooled. Small amount of fresh molten paraplast was poured into the mold, and then the tissue was carefully

transferred onto the paraplast ensuring proper tissue orientation. The mold was filled with the molten paraplast and allowed to cool to room temperature. The cooled paraplast block was attached to a wooden block to allow attachment to the microtome block holder for sectioning.

3.7.1.5 Trimming

The tissues were cut into small pieces to enable quick penetration of reagents during tissue processing. Rotary Microtome (Leica Leitz 1512, United States of America) was used in trimming and sectioning blocks of tissues to obtain thin films of 2-5 microns in thickness which could take in stains for microscopic examination. The knife was firmly fixed on the knife holder and closed tissue block fixed in the rotary arm. The block holder was adjusted using course adjustment moving into the knife and the wheel was moved to start sectioning. The sections were picked using a clean slide with the aid of an applicator stick ensuring the sections lie flat on the slide. Egg albumin served as an adhesive so that the sections do not slide off the slide. 20% alcohol was poured through the slide of the section to flatten the section on the slide.

3.7.1.6 Dewaxing and staining

The sections were stained using haematoxylin and eosin (H & E) method of staining. The sections were de-waxed in two changes of xylene for two minutes each. Xylene was removed in two changes of absolute alcohol for one minute each. The sections were treated with 70% alcohol then washed in tap water. They were then stained with haematoxylin for 20 minutes, and then washed in running tap water. The sections were differentiated in 1% acid alcohol for a few seconds then washed in tap water. This was followed by staining the sections in 1% Eosin for 5 minutes. Excess of the stain was washed off in tap water. The sections were dehydrated in alcohol three times then

cleared in two changes of xylene. A mixture of distyrene, a plasticizer and xylene (DPX) was used in mounting sections. One hundred and twenty labeled slides, two for each tissue, were then placed in a slide tray, and with the matched specimen form, given to the histopathologist for analysis. The histopathologist who examined the slides was "blinded" with respect to the animal identities and treatments to avoid bias in the interpretation of the results.

3.7.2 Photomicrography

An Olympus CX2 binocular microscope was used in the examination of the stained mounted slides. The field of interest was viewed using magnification of X10 and X40 for each of the slides. The images of these tissues were captured using Samsung DV150 digital camera. The field images were then fed into a computer for quantitative image assessing, labeling and printing. Results from microscopic examination of the testes, epididymis, liver, pituitary gland and the hypothalamus were presented as photographic micrographs.

3.8 Data Analysis and Presentation

Data was analyzed using software statistical package for the social sciences (SPSS), the IBM SPSS Statistics version 20. The descriptive analyses for the data were the mean and standard deviations for parameters including body weight, testicular volume and haematological indices. Statistical differences within and between the study groups were determined using one way ANOVA test and Fisher's least significant difference. ANOVA was preferred in statistical analysis of data in this study because the experiment involved just one independent variable and four dependent variables to compare. Results were presented on tables. The metrics analyzed in all the parameters were the mean values of all the animals in each specific group, not the individual

measurements. Statistical values of P<0.05 were considered significant. Histological tissues were examined using Olympus CX 2 binocular microscope. The histological changes were viewed under two different magnifications that is: x40 and x10. This is because some features could not be seen clearly under lower magnification while for other features, use of higher magnification lowered the resolution. The photomicrographs were taken using Samsung DV150 digital camera, labeled and presented.

CHAPTER FOUR

RESULTS

4.1. Effects of Aflatoxin B_1 on Body Weight and Testicular volume.

4.1.1 Effects of varied aflatoxin B_1 levels on body weight.

Oral administration of 80ppb, 160 ppb and 240 ppb AFB_1 levels to pigs for duration of 60 days is shown to have had no significant effect on the animals' body weight. Table 4.1 shows a mean difference of 2.5875 between the control group and the first treated group (p=0.087) and a mean difference of 3.31250 between the control group and the second treated group (p=0.071). The mean difference between the control group and the third treated group is shown to be 0.175 (p=0.905). Thus the aflatoxin B_1 levels administered had no significant effects on body weight of the experimental animals (table 4.1).

Table 4.1: Statistical difference in body weight between the groups using Fisher's least significant difference (LSD)

Multiple Comparisons				
Dependent Variable: Body weight (kg)				
LSD				
(I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
Control	group 1	2.5875	1.4598	0.087
	group 2	3.31250	1.4598	0.071
	group 3	0.175	1.4598	0.905
Group 1	group 2	0.725	1.4598	0.623
	group 3	-2.4125	1.4598	0.11
Group 2	group 3	-3.13750	1.4598	0.06
* The mean difference is significant at the 0.05 level.				

The three levels of AFB_1 administered did not show significant effect on the animals' body weight throughout the experimental period as shown by p=0.068. The control group had a mean body weight of 59.5 (SD 3.17175) which is slightly greater than the mean body weight of group 1 (mean = 56.9125, SD = 2.72105) and group II (mean= 56.1875, SD= 2.14971), and slightly higher than that of the third treated group (mean = 59.325, SD=3.46565) as shown on table 4.2. However, this mean difference was not statistically significant as shown by F=2.65, p=0.068 (table 4.2)

Table 4.2: Statistical difference in body weight within the groups

Body weight (kg)	N	Mean	Standard Deviation	Standard Error	Minimum	Maximum	F	sig.
Control	8	59.5	3.17175	1.12138	53.7	63.5	2.65	0.068
Group 1	8	56.9125	2.72105	0.96204	53.7	60.8		
Group 2	8	56.1875	2.14971	0.76004	53.7	59.4		
Group 3	8	59.325	3.46565	1.22529	53.7	63.7		
Total	32	57.9812	3.14411	0.55581	53.7	63.7		
*** The mean difference is significant at the 0.05 level.**								

4.1.2 Effects of aflatoxin B_1 on testicular volume

Treatment of the experimental pigs with 80ppb, 160 ppb and 240 ppb AFB_1 levels for 60 days caused marked reduction in testicular volumes as evidenced by decreased testicular volumes in the second (160 ppb) and third (240 ppb) treated groups. While the statistical mean difference in testicular volume between the control group and the first treated group (80 ppb) was not significant (p=0.08), the mean differences between the control group and second treated group (mean difference =583.96875) was statistically significant (p=0.05). Notably, the mean difference in testicular volume

between the control group and the third treated group (240ppb) was significant (p=0). These statistical values show a dose-dependent reduction in testicular volumes (Table 4.3).

Table 4.3: Statistical difference in testicular volume in four groups using Fisher's least significant difference (LSD)

Multiple Comparisons				
Dependent Variable: Testicular volume (cm^3)				
LSD				
(I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
control	group 1	546.61750	190.27	0.08
	group 2	583.96875*	190.27	0.05
	group 3	887.28000*	190.27	0
group 1	group 2	37.35125	190.27	0.846
	group 3	340.6625	190.27	0.084
group 2	group 3	303.3113	190.27	0.122
*** The mean difference is significant at the 0.05 level.**				

Testicular volume of the experimental animals declined significantly (F=7.534, p=0.001) in the aflatoxin B_1-treated animals (table 4.4).

Table 4.4: Statistical mean difference in testicular volume within the groups

	N	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**	**F**	**Sig.**
Control	8	2046.02	643.7463	1068.19	3027.47	7.534	0.001
Group 1	8	1499.402	271.4755	1068.19	1875.3		
Group 2	8	1462.051	274.7917	1068.19	1878.64		
Group 3	8	1158.74	124.9953	1068.19	1306.6		
Total	32	1541.553	486.1879	938.09	3027.47		
*** The mean difference is significant at the 0.05 level.**							

4.2 Effects of Aflatoxin B_1 on Haematological Indices

4.2.1 Effects of AFB_1 on white blood cells (WBC's).

Total white blood cell count was altered by the administration of higher levels (240ppb) of AFB_1. The mean differences between the control group and first (80 ppb) treated group (mean difference= 0.6224) as well as the second (160 ppb) treated group (mean difference=0.95846) were not statistically significant (p=0.492 and p=0.292 respectively). On the other hand, the mean difference in white blood cell count between the control group and the third (240 ppb) treated group (240ppb) was 1.88496 which was statistically significant at 0.042 as shown on table 4.5.

Table 4.5: Statistical difference in WBC's in four groups using Fisher's Least Significant Difference.

Multiple Comparisons				
Dependent Variable: WBC(x10^3/µl)				
LSD				
(I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
control	group 1	-0.62224	0.8955	0.492
	group 2	0.95846	0.8955	0.292
	group 3	1.88496*	0.8955	0.042
group 1	group 2	1.5807	0.8955	0.086
	group 3	2.50720*	0.8955	0.008
group 2	group 3	0.9265	0.8955	0.308
*** The mean difference is significant at the 0.05 level.**				

White blood cell count within the groups varied significantly during the study period. The control group indicated the highest mean (Mean= 12.8698, SD= 2.05356) while group three (240 ppb) had the lowest mean of 10.9848, SD=1.549098 as shown on table 4.6. The results demonstrated a significant decrease in white blood cell count

throughout the experimental period following oral administration of aflatoxin B_1 as shown by F=3.014, p= 0.043 (table 4.6).

Table 4.6: Statistical mean difference in white blood cells (WBC's) within the groups

	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
Control	10	12.8698	2.05356	10.43	17.63	3.014	0.043
Group 1	10	13.492	2.03171	11.43	17.63		
Group 2	10	11.9113	2.30622	8.37	14.97		
Group 3	10	10.9848	1.54098	8.17	13.53		
Total	40	12.3145	2.15192	8.17	17.63		
*** The mean difference is significant at the 0.05 level.**							

4.2.2 Effects of AFB_1 on red blood cells (RBCs)

Administration of 80 ppb, 160 ppb and 240 ppb AFB_1 levels to the experimental pigs for a period of 60 days caused a dose-related change in red blood cell count. Multiple comparison of RBC mean differences between the control pigs and the AFB_1 treated pigs showed that the mean difference between the control and first (80 ppb) treated group (mean difference=1.00898) was not statistically significant (p=0.225), while the mean difference between the control group and the second (160 ppb) treated group (mean difference=1.80900) was statistically significant (p=0.033). The mean difference between the control group and the third (240 ppb) treated group was 2.93960 and it was significant (p=0.001). Thus, red blood cell count decreased significantly with increase in AFB_1 levels (table 4.7).

Table 4.7: Statistical values obtained for RBCs in four groups using Fisher's Least Significant Difference.

Multiple Comparisons				
Dependent Variable: RBC (x10^6/µl)				
LSD				
(I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
control	group 1	1.00898	0.81686	0.225
	group 2	1.80900*	0.81686	0.033
	group 3	2.93960*	0.81686	0.001
group 1	group 2	0.80002	0.81686	0.334
	group 3	1.93062*	0.81686	0.024
group 2	group 3	1.1306	0.81686	0.175
* The mean difference is significant at the 0.05 level.				

Red blood cell count declined significantly (F=4.64, P= 0.008) as shown on table 4.8 following oral administration of 80pp, 160 ppb and 240 ppb aflatoxin B_1 levels for 60 days. The results showed a significant decrease in RBC count in AFB_1 treated animals throughout the entire experimental period.

Table 4.8: Statistical mean difference of RBCs within the control and the treated groups

RBC ((x10^6/µl)	**N**	**Mean**	**Std. Deviation**	**Minimum**	**Maximum**	**F**	**Sig.**
Control	10	10.1684	2.35821	7.8	13.51	4.64	0.008
Group 1	10	9.1594	2.26134	5.66	13.38		
Group 2	10	8.3594	1.14363	6.97	10.32		
Group 3	10	7.2288	1.16728	5.19	9.51		
Total	40	8.729	2.06653	5.19	13.51		
*** The mean difference is significant at the 0.05 level.**							

4.2.3 Effects of AFB_1 on haemoglobin (Hb)

Following oral administration of 80 ppb, 160 ppb and 240 ppb AFB_1 levels, haemoglobin concentration was noted to reduce significantly in the all the treated animals. The mean difference between the control and first (80 ppb) treated group (mean difference=2.27225) was statistically significant as the P value obtained was 0.026. The mean difference between the control group and the second (160 ppb) treated group (mean difference= 2.83690) was shown to be highly significant (p=0.006). The mean difference in haemoglobin concentration between the control group and the third (240 ppb) treated group (mean difference=4.21610) was very highly significant (p=0.000). Aflatoxin B_1 levels administered were shown to cause significant decrease in haemoglobin in the experimental animals (table 4.9).

Table 4.9: Statistical difference of haemoglobin in four groups using Fisher's Least Significant Difference

Multiple Comparisons				
Dependent Variable: Hb (g/dl)				
LSD				
(I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
control	group 1	2.27225*	0.97669	0.026
	group 2	2.83690*	0.97669	0.006
	group 3	4.21610*	0.97669	0.000
group 1	group 2	0.56465	0.97669	0.567
	group 3	1.94385	0.97669	0.054
group 2	group 3	1.3792	0.97669	0.167
*** The mean difference is significant at the 0.05 level.**				

Oral administration of AFB_1 resulted in statistically significant decline in haemoglobin concentration in the AFB_1 challenged animals (table 4.10). These results showed a significant decrease in haemoglobin concentration (F=6.462, p=0.001) in the aflatoxin B_1 treated pigs throughout the experimental period (table 4.10).

Table 4.10: Statistical mean difference of Hb within the control and within the AFB_1-treated groups

Hb (g/dl)	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	10	12.9766	2.24814	9.47	15.93	6.462	0.001
group 1	10	10.7043	2.72578	6.47	15.97		
group 2	10	10.1397	1.74287	7.9	13.63		
group 3	10	8.7605	1.88601	6.23	11.1		
Total	40	10.6453	2.60263	6.23	15.97		

*** The mean difference is significant at the 0.05 level**

4.2.4 Effects of AFB_1 on mean corpuscular volume (MCV)

Administration of 80 ppb, 160 ppb and 240 ppb AFB_1 levels resulted in increased mean corpuscular volume in all the AFB_1-treated animals. However, only the third (240 ppb) treated group showed a statistically significant increase in MCV (p= 0.002) as shown on table 4.11. The mean difference between the control group and the first treated group which received 80 ppb AFB_1 level (mean difference=1.2031) was not statistically significant as shown by P value of 0.196. The mean difference between the control group and the second treated group which received 160 ppb AFB_1 level (mean difference=1.5699) was not statistically significant (p=0.094). However, the mean difference in MCV between the control group and the third treated group which received 240 ppb group (mean difference= 3.07684) was highly significant as evidenced by p value of 0.002. This shows that the third treated group experienced a significant increase in the MCV level compared to the second and first AFB_1- treated groups (table 4.11).

Table 4.11: Mean differences in MCV between the control group and the AFB_1 treated groups using Fisher's Least Significant difference (LSD)

Multiple Comparisons Dependent Variable: MVC (fl)						
LSD (I) treatment	**(J) treatment**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**95% Confidence Interval**	
control	group 1	-1.2031	0.91309	0.196	-3.0549	0.6487
	group 2	-1.5699	0.91309	0.094	-3.4217	0.2819
	group 3	-3.07684*	0.91309	0.002	-4.9287	-1.225
group 1	group 2	-0.3668	0.91309	0.69	-2.2186	1.485
	group 3	-1.87374*	0.91309	0.047	-3.7256	-0.0219
group 2	group 3	-1.50694	0.91309	0.108	-3.3588	0.3449

*** The mean difference is significant at the 0.05 level.**

The plasma mean corpuscular volume increased significantly ($F=3.857$, $p=0.017$) following the oral administration of aflatoxin B_1 to the experimental animals (table 4.12). These results demonstrated a significant increase in plasma MCV throughout the experimental period in the aflatoxin B_1 treated animals.

Table 4.12: Shows the mean difference in MCV within the four groups

MCV (fl)	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	10	55.8604	1.82584	52.33	57.83	3.857	0.017
group 1	10	57.0635	1.7037	53.67	60.33		
group 2	10	57.4303	1.5872	53.67	59.6		
group 3	10	58.9372	2.8141	53.67	64.8		
Total	40	57.3229	2.25496	52.33	64.8		

*** The mean difference is significant at the 0.05 level.**

4.2.5 Effects of AFB_1 on mean corpuscular haemoglobin (MCH)

MCH increased in all the animals treated with AFB_1 compared to the control group. This is evidenced by the negative mean differences between the control group and the treated groups. However, only the mean difference between the control group and the 240 ppb group (mean difference=1.32798) was statistically significant as shown by p=0.007. Thus the effect of AFB_1 on mean corpuscular haemoglobin was dependent on the toxin level administered in that 240 ppb caused a statistically significant increase in the mean corpuscular haemoglobin compared to the 80 ppb AFB_1 levels (table 4.13).

Table 4.13: Mean differences in MCH between the control group and the AFB_1-treated groups using Fisher's Least Significant difference (LSD)

Multiple Comparisons						
Dependent Variable: MCH (pg)						
LSD						
(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
control	group 1	-0.54656	0.4612	0.244	-1.4819	0.3888
	group 2	-0.66999	0.4612	0.155	-1.6054	0.2654
	group 3	-1.32798*	0.4612	0.007	-2.2633	-0.3926
group 1	group 2	-0.12343	0.4612	0.791	-1.0588	0.8119
	group 3	-0.78142	0.4612	0.099	-1.7168	0.1539
group 2	group 3	-0.65799	0.4612	0.162	-1.5934	0.2774
* The mean difference is significant at the 0.05 level.						

The mean corpuscular haemoglobin in the plasma of the AFB_1 treated animals was noted to increase significantly (F=3.797, p= 0.047) following administration of 240 ppb AFB_1level. The results showed a demonstrated a significant increase in MCH in the plasma of the experimental animals during the 60 days period of experiment (table 4.14)

Table 4.14: Shows the mean difference in MCH within the four groups

MCH (pg)	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	10	11.5708	0.32308	11.23	12.37	3.797	0.047
group 1	10	12.1174	0.79715	11.34	13.67		
group 2	10	12.2408	0.96213	11.2	13.87		
group 3	10	12.8988	1.60892	11.34	16.43		
Total	40	12.207	1.10026	11.2	16.43		

4.2.6 Effects of AFB_1 on mean corpuscular haemoglobin concentration (MCHC)

Mean corpuscular haemoglobin concentration decreased significantly in all AFB_1 treated animals compared to the control animals (table 4.15).

Table 4.15: Mean differences in MCHC between the control group and the treated groups using Fisher's Least Significant difference (LSD)

Multiple Comparisons						
Dependent Variable: MCHC (g/dl)						
LSD						
(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
control	group 1	1.96324*	0.37221	0	1.2084	2.7181
	group 2	2.16765*	0.37221	0	1.4128	2.9225
	group 3	2.42329*	0.37221	0	1.6684	3.1782
group 1	group 2	0.20441	0.37221	0.586	-0.5505	0.9593
	group 3	0.46005	0.37221	0.224	-0.2948	1.2149
group 2	group 3	0.25564	0.37221	0.497	-0.4992	1.0105
* The mean difference is significant at the 0.05 level.						

The plasma mean corpuscular haemoglobin concentration was shown to decrease significantly (F=17.738, p=0) in all AFB_1 treated animals following oral administration of 80ppb, 160 ppb and 240 ppb respectively for 60 days (table 4.16)

Table 4.16: Statistical mean differences in MCHC within the control group and the experimental groups

MCHC (g/dl)	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	10	22.3125	1.20372	21.13	24.63	17.738	0
group 1	10	20.3492	0.64165	19.1	21.23		
group 2	10	20.1448	0.73869	18.72	21.23		
group 3	10	19.8892	0.60368	19.13	21.23		
Total	40	20.6739	1.25879	18.72	24.63		

* The mean difference is significant at the 0.05 level.

4.2.7 Effects of AFB_1 on Platelets

Platelet count was not altered by the 80 ppb, 160 ppb and 240 ppb AFB_1 levels administered to the animals for a period of 60 days. This is shown by comparison of the statistical mean differences in platelet count between the control group and the AFB_1 challenged groups. Table 4.17 shows that all the AFB_1 treated pigs had a slightly increased number of platelets compared to the control pigs however, these mean differences were not statistically significant as shown by p=0.313, p=0.436 and p=0.063 respectively (table 4.17).

Table 4.17: Statistical mean differences in platelet count between the control group and the experimental groups using Fisher's Least Significant Difference (LSD)

Multiple Comparisons						
Dependent Variable: Platelets (x10^3/µl)						
LSD						
(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
control	group 1	-71.8325	70.18041	0.313	-214.165	70.5
	group 2	-55.2332	70.18041	0.436	-197.566	87.0993
	group 3	-96.2412	70.18041	0.063	-324.889	-40.2242
group 1	group 2	16.59927	70.18041	0.814	-125.733	158.9317
	group 3	-110.724	70.18041	0.123	-253.057	31.6082
group 2	group 3	-127.324	70.18041	0.078	-269.656	15.009
* The mean difference is significant at the 0.05 level.						

There was no significant effect in platelet count (F=2.378, p=0.086) in all the aflatoxin B_1- treated animals following oral administration of 80ppb, 160 ppb and 240 ppb AFB_1 levels for the entire experimental period (table 4.18).

Table 4.18: Statistical mean difference in platelet count within the control group and the experimental groups

Platelets (x10^3/µl)	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	10	459.075	94.02377	309	620	2.378	0.086
group 1	10	530.9075	183.336	325.66	861.33		
group 2	10	514.3082	137.7477	367	734.33		
group 3	10	641.6317	192.5586	390.33	934		
Total	40	536.4806	165.0379	309	934		

* The mean difference is significant at the 0.05 level.

4.2.8 Effects of AFB_1 on granulocytes

Administration of the different AFB_1 levels had no significant effect on granulocytes. The mean differences within the groups was not statistically significant as shown by F=0.105, p= 0.957

(table 4.19).

Table 4.19: Statistical mean difference in granulocytes within the control group and the experimental groups

	N	Mean	Std. Deviation	Std. Error	F	Sig.
control	30	4.55	1.501	0.274	0.105	0.957
group 1	30	4.33	2.07	0.378		
group 2	30	4.55	2.113	0.386		
group 3	30	4.44	1.399	0.255		
Total	120	4.47	1.779	0.162		

Mean differences in granulocyte count between the control group and the three experimental groups were not statistically significant. The mean difference between the control group and the first treated group was p=0.637, between the control group and the second treated group was p=0.994 while the mean difference between the control and third treated group was p=0.808 as shown on table 4.20

Table 4.20: Statistical mean differences in granulocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD)

Multiple Comparisons				
Dependent Variable: Granulocytes (x10^3/µl)				
LSD				
(I) groups	(J) groups	Mean Difference (I-J)	Std. Error	Sig.
control	group 1	0.22	0.465	0.637
	group 2	-0.003	0.465	0.994
	group 3	0.113	0.465	0.808
group 1	group 2	-0.223	0.465	0.632
	group 3	-0.107	0.465	0.819
group 2	group 3	0.117	0.465	0.802

4.2.9 Effects of AFB_1 on lymphocytes

The aflatoxin B_1 levels administered had a statistically significant effect on the lymphocytes. This effect was not dose-dependent since the mean differences between the control group and the three treated groups was statistically significant p=0 as shown on table 4.21

Table 4.21: Statistical mean differences in lymphocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD)

Multiple Comparisons				
Dependent Variable: Lymphocytes ($x10^3/\mu l$)				
LSD				
(I) groups	**(J) groups**	**Mean Difference (I-J)**	**Std. Error**	**Sig.**
control	group 1	2.12000*	0.5284	0.0
	group 2	4.19333*	0.5284	0
	group 3	5.66333*	0.5284	0
group 1	group 2	2.07333*	0.5284	0
	group 3	3.54333*	0.5284	0
group 2	group 3	1.47000*	0.5284	0.006
*** The mean difference is significant at the 0.05 level.**				

4.3 Effects of AFB_1 on Monocytes

The three aflatoxin B_1 levels administered had a statistically significant effect on the monocytes. This is shown by the mean differences between the control group and the three treated groups with a p value of zero. The effects of AFB_1 on monocytes were not dose-dependent as shown on table 4.22.

Table 4.22: Statistical mean differences in monocytes between the control group and the experimental groups using Fisher's Least Significant Difference (LSD)

Multiple Comparisons				
Dependent Variable: Monocytes ($x10^3/\mu l$)				
LSD				
(I) groups	(J) groups	Mean Difference (I-J)	Std. Error	Sig.
control	group 1	1.06333*	0.15932	0
	group 2	1.17000*	0.15932	0
	group 3	1.60000*	0.15932	0
group 1	group 2	0.10667	0.15932	0.504
	group 3	.53667*	0.15932	0.001
group 2	group 3	.43000*	0.15932	0.008
* The mean difference is significant at the 0.05 level.				

4.4 Effects of Aflatoxin B_1 ON FSH, LH, TH AND E2 Hormone Levels

4.4.1 Porcine follicle stimulating hormone (FSH)

Oral administration of 80ppb, 160 ppb and 240 ppb AFB_1 levels had no significant effect on follicle stimulating hormone concentration. Although the mean differences in FSH concentrations between the control group and the AFB_1 treated groups showed that FSH levels were slightly higher in the AFB_1 treated groups (group I=1.04323; group II =1.46347; group III =1.62406), these mean differences were not statistically significant as evidenced by p values of 0.203, 0.079 and 0.063 respectively. This implies that the treatment did not cause a significant alteration of the FSH levels in either of the treated groups (table 4.23).

Table 4.23: Statistical mean differences in the concentration of FSH between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference (LSD)

Dependent Variable: FSH concentration					
LSD	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.
	control	group 1	-1.04323	0.79635	0.203
		group 2	-1.46347	0.79635	0.079
		group 3	-1.62406	0.79635	0.063
	group 1	group 2	-0.16059	0.79635	0.842
		group 3	0.42024	0.79635	0.603
	group 2	group 3	0.58083	0.79635	0.473
	group 3	control	1.04323	0.79635	0.203

* The mean difference is significant at the 0.05 level

The mean differences in the concentration of FSH within the groups were not statistically significant (F=1.684, p=0.197). These results showed that the AFB_1 levels administered did not significantly affect FSH concentration for the entire experimental period (table 4.24).

Table 4.24: Statistical difference in FSH concentration within the control group and within the AFB_1-treated groups

FSH conc.							
	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
Control	7	5.9796	1.23515	4.39	7.87	1.684	0.197
Group 1	7	7.4431	0.89756	5.94	8.36		
Group 2	7	7.6037	1.97629	4.93	11.16		
Group 3	7	7.0229	1.62529	5.21	9.8		
Total	28	7.0123	1.54541	4.39	11.16		

*** The mean difference is significant at the 0.05 level.**

4.4.2 Porcine Luteinizing Hormone (LH)

Administration of 80 ppb, 160 ppb and 240 ppb AFB_1 levels resulted in changes in luteinizing hormone concentration. There was no significant difference in LH concentration between the control group and the first treated group (80 ppb) or the second treated group (160 ppb) as shown on table 4.21. The third (240 ppb) treated group was shown to have a lower mean with a difference of 1.73641 compared to the control group and a statistical significance of (p=0.042). Thus the luteinizing hormone concentration in the third treated group (240 ppb) was significantly reduced as compared to the control group. This shows that the effect of AFB_1 on LH was dependent on AFB_1 level administered. Luteinizing hormone concentration decreased significantly with increased aflatoxin B_1 levels as shown on table 4.25

Table 4.25: Statistical mean differences in the concentration of LH between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference.

Multiple Comparisons				
Dependent Variable: LH concentration				
LSD				
(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.
control	group 1	-1.62449	0.83203	0.063
	group 2	-0.40781	0.83203	0.057
	group 3	1.73641*	0.83203	0.042
	control	1.62449	0.83203	0.063
group 1	group 2	1.21668	0.83203	0.157
	group 3	1.72011*	0.83203	0.050
group 2	group 3	0.50343	0.83203	0.551
* The mean difference is significant at the 0.05 level.				

Analysis of the statistical mean differences within the control group and the AFB_1 challenged groups showed that the LH mean differences within each of the groups was

statistically significant as shown by F=3.807, p= 0.042 (table 4.26). These results demonstrated that administration of the three AFB_1 levels for an experimental period of 60 days significantly reduced luteinizing hormone concentration in the plasma of the AFB_1-treated animals.

Table 4.26: Statistical mean differences in LH concentration within the control group and within the treated groups

LH concentration							
	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	7	3.9699	0.46607	3.19	9.87	3.807	0.042
group 1	7	5.5944	2.01388	3.71	9.57		
group 2	7	4.3777	1.71775	2.38	7.57		
group 3	7	3.8742	1.57107	2.81	6.19		
Total	28	4.454	1.6249	2.38	9.87		

*** The mean difference is significant at the 0.05 level.**

4.4.3 Porcine Testosterone Hormone

Treatment of reproductively mature male pigs with 80 ppb, 160 ppb and 240 ppb AFB_1 levels for a period of 60 days resulted in decreased concentration in testosterone hormone. Comparison of the statistical mean differences between the control group and the AFB_1 challenged groups revealed that from the control group to the first (80 ppb) treated group, the mean difference was zero and statistically insignificant (p=1). The mean differences between the control group and the second (160 ppb) and third (240 ppb) treated groups were statistically significant with a mean difference of 73.28571 and 87.14286 respectively and p values of 0.009 and 0.002 respectively as shown on table 4.27. The results showed that the second treated group and the third treated groups had significantly reduced testosterone levels compared to the control group. This shows a dose-related effect of AFB_1 on the testosterone concentration in plasma samples.

Table 4.27: Statistical mean differences in the concentration of testosterone hormone between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference.

Multiple Comparisons					
Dependent Variable: T H conc.					
	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.
LSD	control	group 1	0	25.70043	1
		group 2	73.28571*	25.70043	0.009
		group 3	87.14286*	25.70043	0.002
	group 1	group 2	73.28571*	25.70043	0.009
		group 3	87.14286*	25.70043	0.002
	group 2	group 3	13.85714	25.70043	0.595
*** The mean difference is significant at the 0.05 level.**					

Table 4.24 shows the statistical mean differences in the concentration of testosterone hormone within each individual group for a period of 60 days. The control group and first treated group (80 ppb) recorded the highest mean values of 150.8571 with SD 54.63951 and 37.47229 respectively. The lowest recorded mean value was from the third treated group (240 ppb) (minimum value=0). The second treated group (160 ppb) recorded a maximum mean value of 150 and a minimum mean value of 10 (SD = 53.15924). The mean differences within the groups were statistically significant (F= 6.591, p=0.002). These results demonstrated that the aflatoxin B_1 levels administered caused a significant decrease in testosterone hormone concentration in all the AFB_1-treated animals during the experimental period of 60 days (table 4.28).

Table 4.28: Statistical mean differences in the concentration of testosterone within the control and the within the treated groups

Testosterone concentration							
	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	7	150.8571	54.63951	76	229	6.591	0.002
group 1	7	150.8571	37.47229	76	189		
group 2	7	77.5714	45.07348	10	150		
group 3	7	63.7143	53.15924	0	150		
Total	28	110.75	61.22074	0	229		

4.4.4 Porcine Estradiol (E2) Hormone

Oral administration of 80 ppb, 160 ppb and 240 ppb AFB_1 levels resulted in changes in the concentration of plasma estradiol hormone concentration in the experimental pigs. The mean differences between the control group and each of the AFB_1 challenged groups showed that, from the control group to the first treated group (80 ppb), the mean difference was 3.37143 which was not statistically significant (p=0.752). The mean differences between the control group and the second (160 ppb) treated group (mean=24.8000) was statistically significant as shown by a p value of 0.027. The mean difference between the control group and the third (240 ppb) treated group was higher (mean=40.9000) and was highly significant (p= 0.001).The results showed that the second treated group and the third treated groups had significantly reduced estradiol levels compared to the control group. The results also showed a dose-related effect of AFB_1 on the estradiol concentration in plasma samples of the treated pigs in that E2 decreased with increase in AFB_1 levels (table 4.29).

Table 4.29: Statistical mean differences in the concentration of E2 between the control group and the AFB_1 treated groups using Fisher's Least Significant Difference

Multiple Comparisons							
Dependent Variable: E2 conc.							
	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
LSD	control	group 1	-3.37143	10.53923	0.752	-25.1233	18.3805
		group 2	24.80000*	10.53923	0.027	3.0481	46.5519
		group 3	40.90000*	10.53923	0.001	19.1481	62.6519
	group 1	group 2	28.17143*	10.53923	0.013	6.4195	49.9233
		group 3	44.27143*	10.53923	0	22.5195	66.0233
	group 2	group 3	16.1	10.53923	0.14	-5.6519	37.8519
	* The mean difference is significant at the 0.05 level.						

The maximum mean value for the control group was 107 and a minimum mean value of 31.22 (SD=17.43327). The first treated group (80 ppb) had the highest maximum mean value of 76.4 and a minimum mean value of 31.11 (SD=30.14911). The third treated group recorded the lowest maximum mean value of 37.7 and a low mean value of -10.5 (SD=16.59554). These results showed that the aflatoxin B_1 levels administered significantly ($F=7.971$, $p=0.001$) reduced estradiol hormone concentration in all the AFB_1-treated animals (table 4.30).

Table 4.30: Statistical mean differences in the concentration of E2 within the control and the treated groups

	N	Mean	Std. Deviation	Minimum	Maximum	F	Sig.
control	7	58.8871	17.43327	31.11	107	7.971	0.001
group 1	7	62.2586	30.14911	31.11	76.4		
group 2	7	34.0871	8.17036	23.6	45.9		
group 3	7	17.9871	16.59554	-10.5	47.7		
Total	28	43.305	26.26528	-10.5	107		

4.5 Histopathological Changes Caused By Aflatoxin B_1

4.5.1 Histopathological changes in the testes

Examination of the histological changes in the testes of the control and the AFB_1-treated pigs showed varying results. Testes from the control group showed normal histology (plate 1). Histopathological changes observed in the testes of pigs belonging to the three experimental groups were progressive. The first experimental group (80 ppb) showed mild reduction of spermatogenesis cells and reduced number of Leydig cells (plate 2). The second experimental group (160 ppb) showed moderate reduction in the number of spermatogenesis cells as well as moderate reduction in the number of Leydig cells (plates 3 & 4). In the third experimental group (240 ppb) there was marked loss of spermatogenesis cells within the seminiferous tubules and thus reduction in spermatids, almost complete loss (plate 7 & 8). The number of Leydig cells and Sertoli cells was markedly reduced (plate 9) in the third treated group. There was increased peritubular oedema as well as necrosis of the seminiferous tubules (plate 5 & 6). Atrophy of seminiferous tubules as evidenced by the thickening of the basement membrane was observed in the third (240 ppb) treated group (plate 11). The observed pathological

changes clearly reflected dose dependent effects of aflatoxin B_1 on the testes of male pigs.

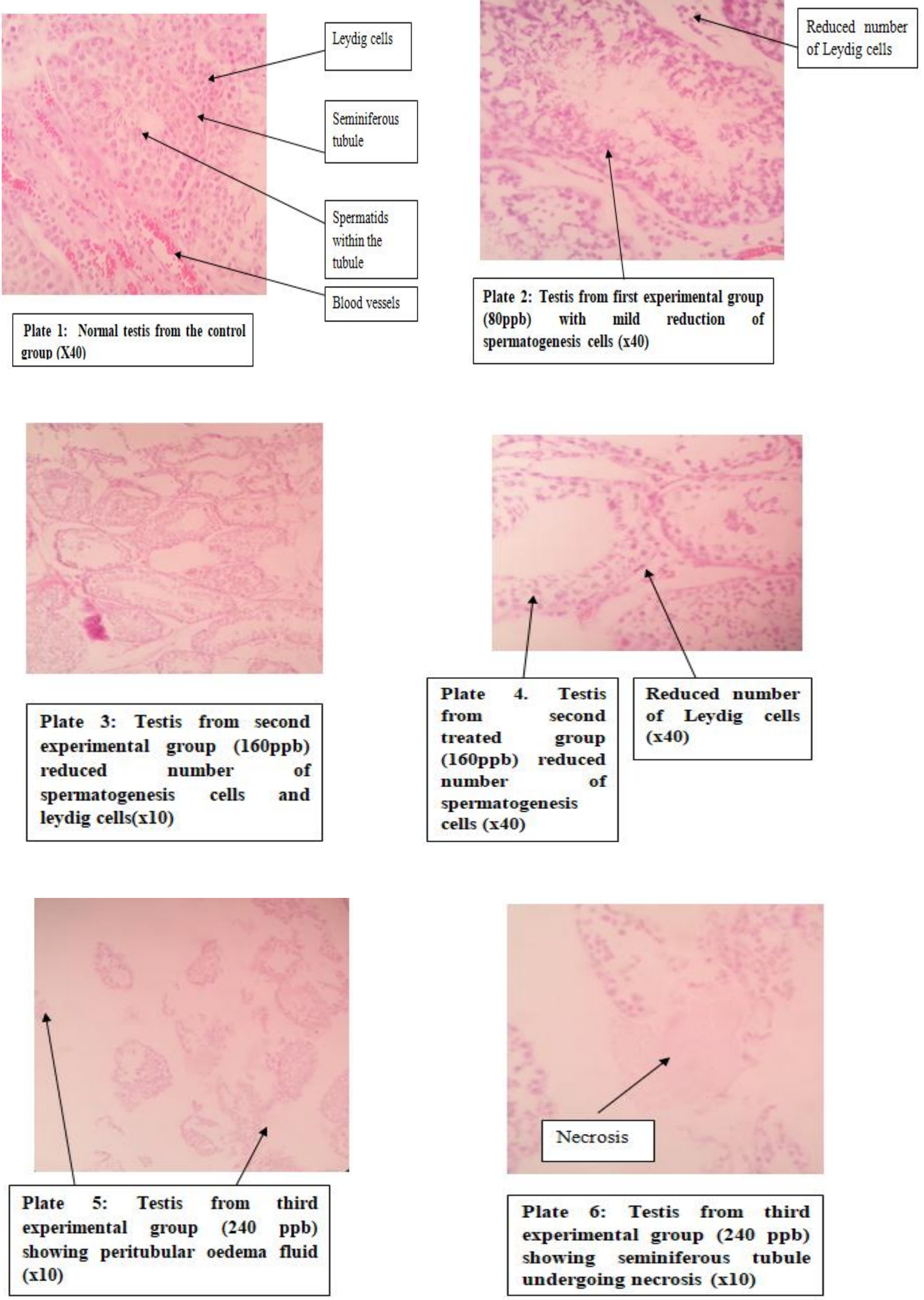


Plate 1: Normal testis from the control group (X40)

Plate 2: Testis from first experimental group (80ppb) with mild reduction of spermatogenesis cells (x40)

Plate 3: Testis from second experimental group (160ppb) reduced number of spermatogenesis cells and leydig cells(x10)

Plate 4. Testis from second treated group (160ppb) reduced number of spermatogenesis cells (x40)

Plate 5: Testis from third experimental group (240 ppb) showing peritubular oedema fluid (x10)

Plate 6: Testis from third experimental group (240 ppb) showing seminiferous tubule undergoing necrosis (x10)

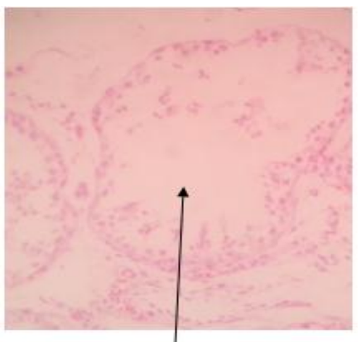


Plate 7: Testis from third experimental group (240ppb) showing loss of spermatogenesis cells within seminiferous tubules (x40)

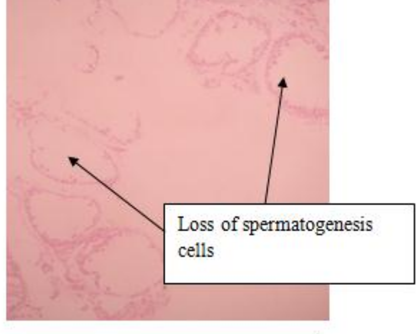


Plate 8: Testis from third experimental group (240ppb) showing loss of spermatogenesis cells within seminiferous tubules (x10)

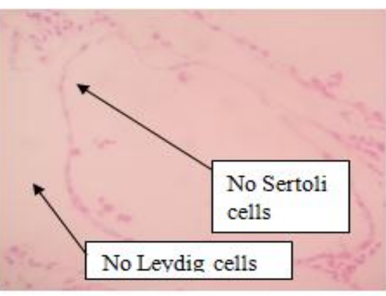


Plate 9: Testis from third experimental group (240ppb) showing loss of Leydig cells and spermatogenesis cells within seminiferous tubules (x40)

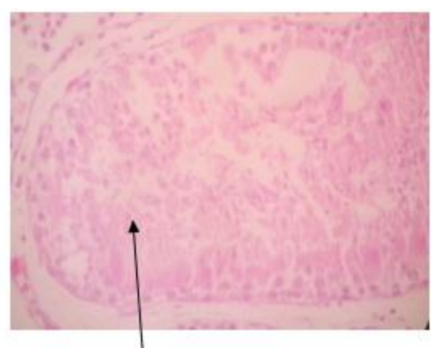


Plate 10: Testis from third experimental group (240ppb) showing degenerating seminiferous tubule (x40)

Plate 11: Atrophic testis from third experimental group (240ppb) showing thickened basement membrane (x40)

Plate 1 to 11: Histopathological changes in the testes

4.4.2 Histopathological changes in the epididymis induced by AFB_1

Microscopic examination of the epididymis of the control group revealed normal histology (plate 12). Epithelial hyperplasia was observed in all the AFB_1 treated groups, an effect which did not seem to relate to the aflatoxin dosage given. This was evident in the 80 ppb group (plate 15), the 160 ppb group (plate 13) and the 240 ppb group (plate 14). Epithelial cell cytoplasmic vacuolation was also observed in the first treated group (plate 15) and in the third treated group (plate 14). Several secondary vacuoles, mainly filled with lipids were seen in the cytoplasm of the epithelial cells of the AFB_1 treated pigs. These vacuoles were mainly located in the basal zone of the epithelium.

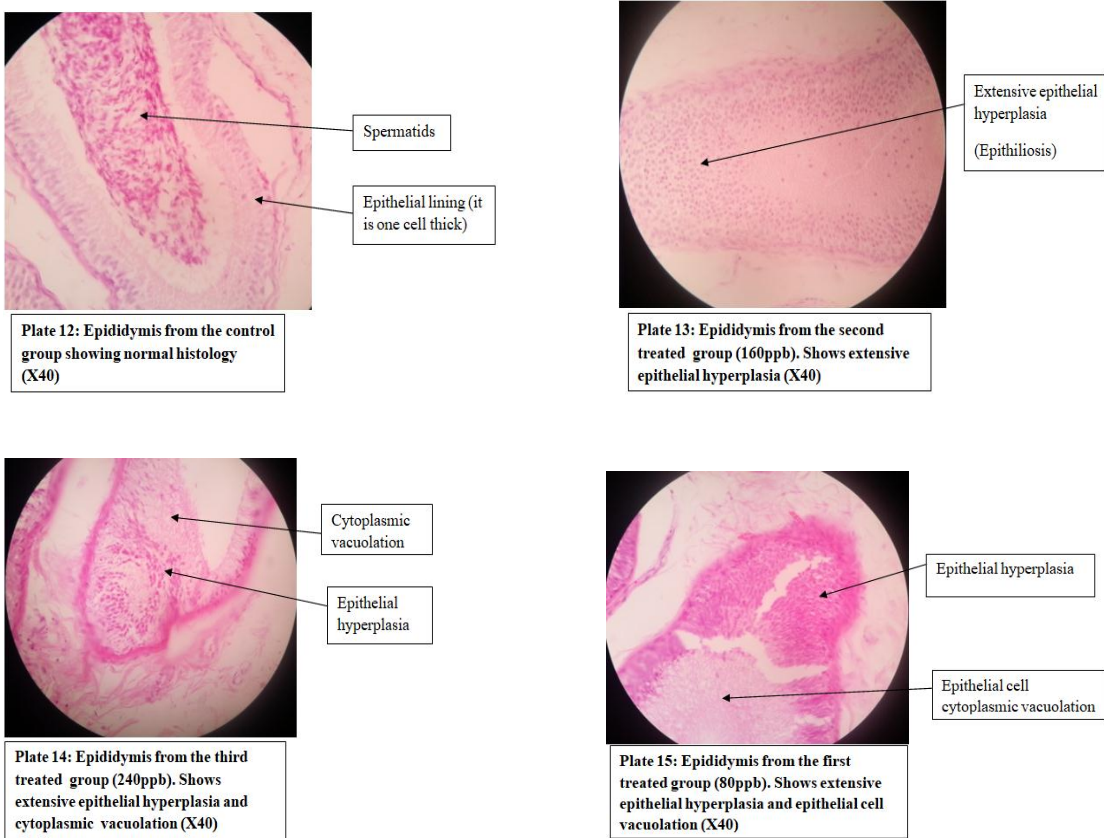


Plate 12: Epididymis from the control group showing normal histology (X40)

Plate 13: Epididymis from the second treated group (160ppb). Shows extensive epithelial hyperplasia (X40)

Plate 14: Epididymis from the third treated group (240ppb). Shows extensive epithelial hyperplasia and cytoplasmic vacuolation (X40)

Plate 15: Epididymis from the first treated group (80ppb). Shows extensive epithelial hyperplasia and epithelial cell vacuolation (X40)

Plate 12 to 15: Histopathological changes in the epididymis

4.4.3 Histopathological changes in the liver induced by AFB_1

Histopathological changes in the liver of the control and treated groups were examined. The histology of liver from the control group appeared normal (plate 16). Significant changes were found in the liver tissues of pigs treated with AFB_1. These changes included marked bile duct proliferation which was observed predominantly in the second and third experimental groups (plates 18, 26, & 27); Dilatation of the central vein which was observed in all the aflatoxin treated groups (plates 19 & 23). Mononuclear cell infiltration of portal area was observed in the second and third treated groups (plates 20 &24). Cytoplasmic vacuolation or fatty change (steatosis) of hepatocytes was observed in both the second and the third treated groups (plate 21). Fibrosis was noted in the third treated group only (plate 25) while the second treated group showed marked congestion of the parenchyma (plate 22). A number of these observations were not dose-dependent for example dilatation of the central vein and bile duct proliferation which were observable in all the treated groups. Both the 160 ppb and 240 ppb treated groups had observable fatty change as well as mononuclear infiltration, a reflection of chronic inflammation.

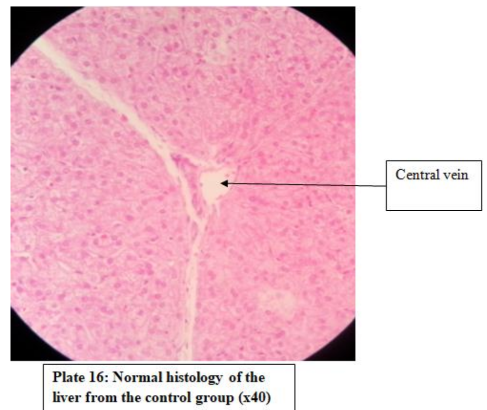


Plate 16: Normal histology of the liver from the control group (x40)

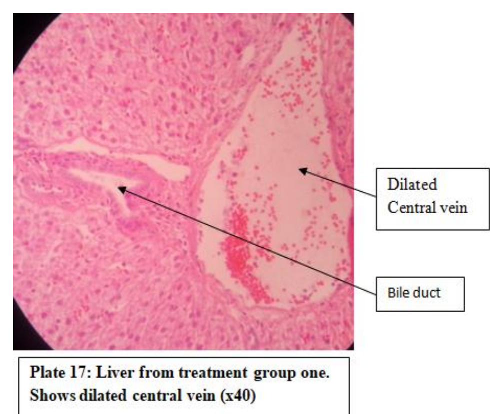


Plate 17: Liver from treatment group one. Shows dilated central vein (x40)

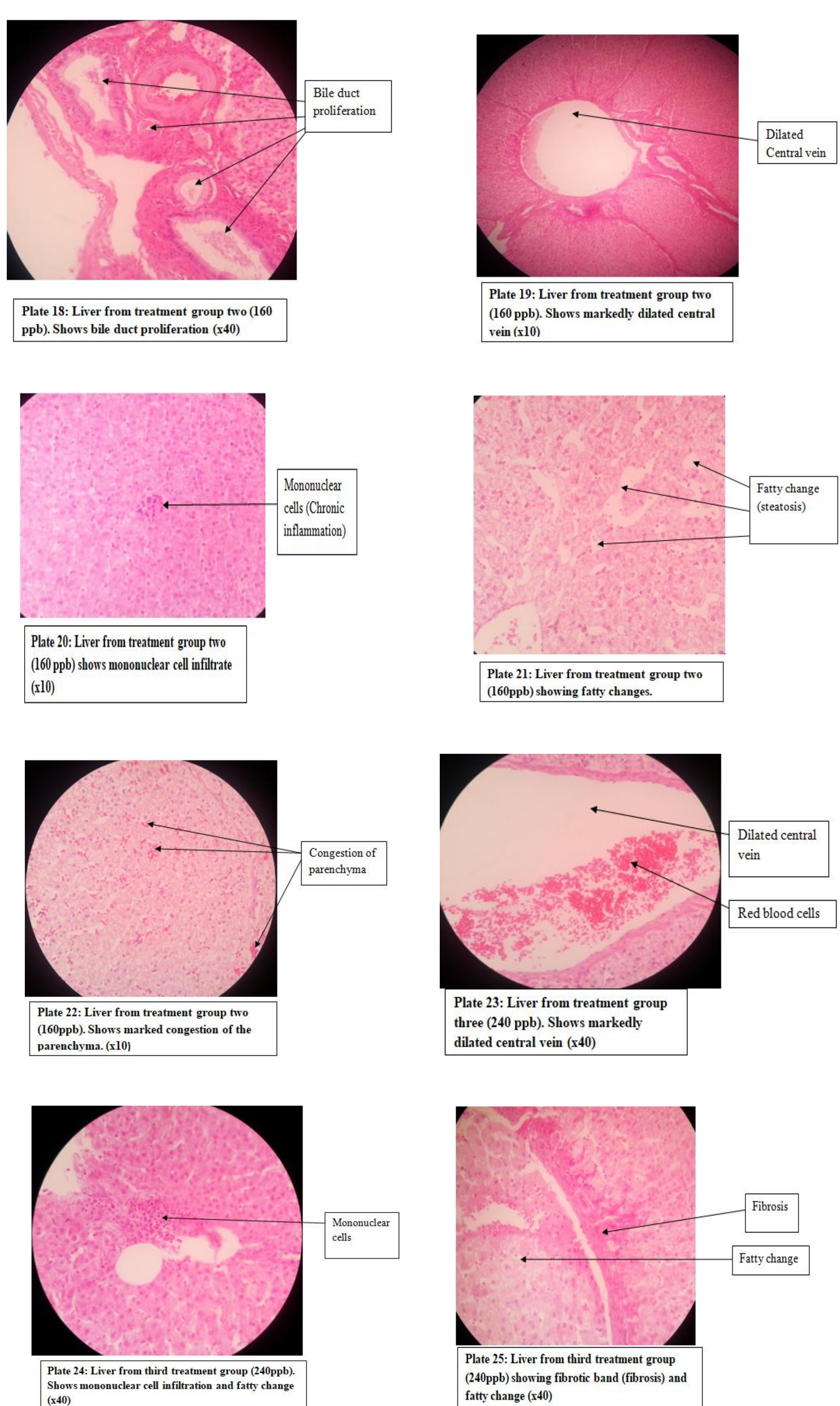


Plate 18: Liver from treatment group two (160 ppb). Shows bile duct proliferation (x40)

Plate 19: Liver from treatment group two (160 ppb). Shows markedly dilated central vein (x10)

Plate 20: Liver from treatment group two (160 ppb) shows mononuclear cell infiltrate (x10)

Plate 21: Liver from treatment group two (160ppb) showing fatty changes.

Plate 22: Liver from treatment group two (160ppb). Shows marked congestion of the parenchyma. (x10)

Plate 23: Liver from treatment group three (240 ppb). Shows markedly dilated central vein (x40)

Plate 24: Liver from third treatment group (240ppb). Shows mononuclear cell infiltration and fatty change (x40)

Plate 25: Liver from third treatment group (240ppb) showing fibrotic band (fibrosis) and fatty change (x40)

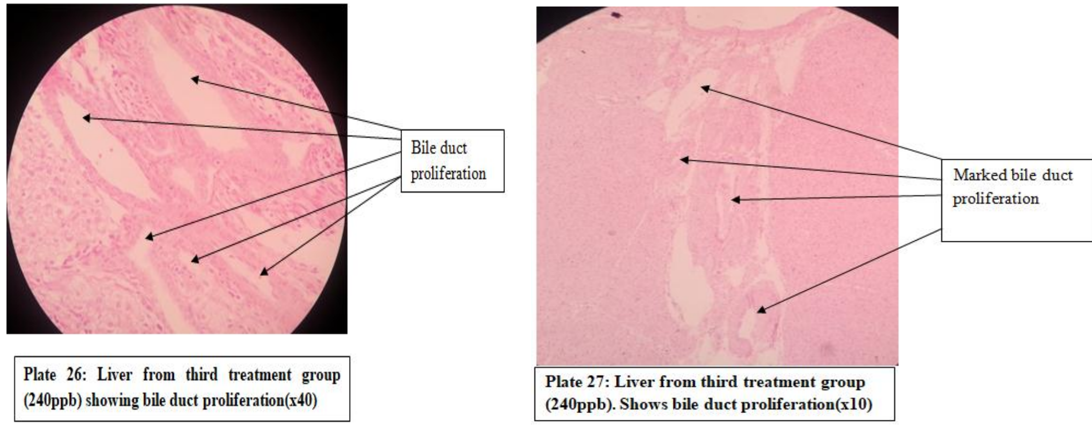


Plate 26: Liver from third treatment group (240ppb) showing bile duct proliferation(x40)

Plate 27: Liver from third treatment group (240ppb). Shows bile duct proliferation(x10)

Plate 16 to 27: Histopathological changes in the liver

4.4.4 Histopathological changes in the hypothalamus and pituitary gland related to AFB_1

Microscopic examination of the hypothalamus and the pituitary gland did not reveal any histopathological change related to aflatoxin B_1 under light microscopy.

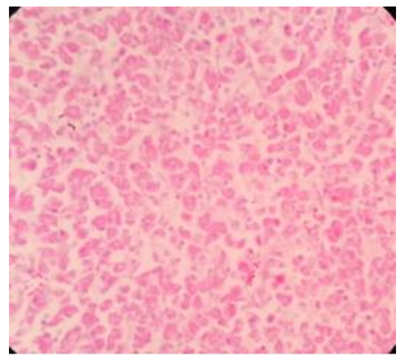


Plate 28: Pituitary gland from control group showing normal histology (X40)

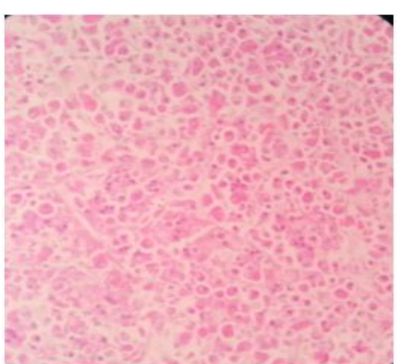


Plate 29: Pituitary gland from first treated group (80ppb) showing normal histology (X40)

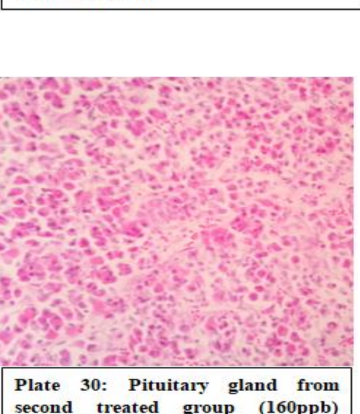


Plate 30: Pituitary gland from second treated group (160ppb) showing normal histology (X40)

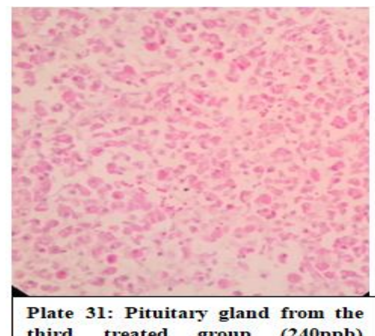


Plate 31: Pituitary gland from the third treated group (240ppb) showing normal histology (X40)

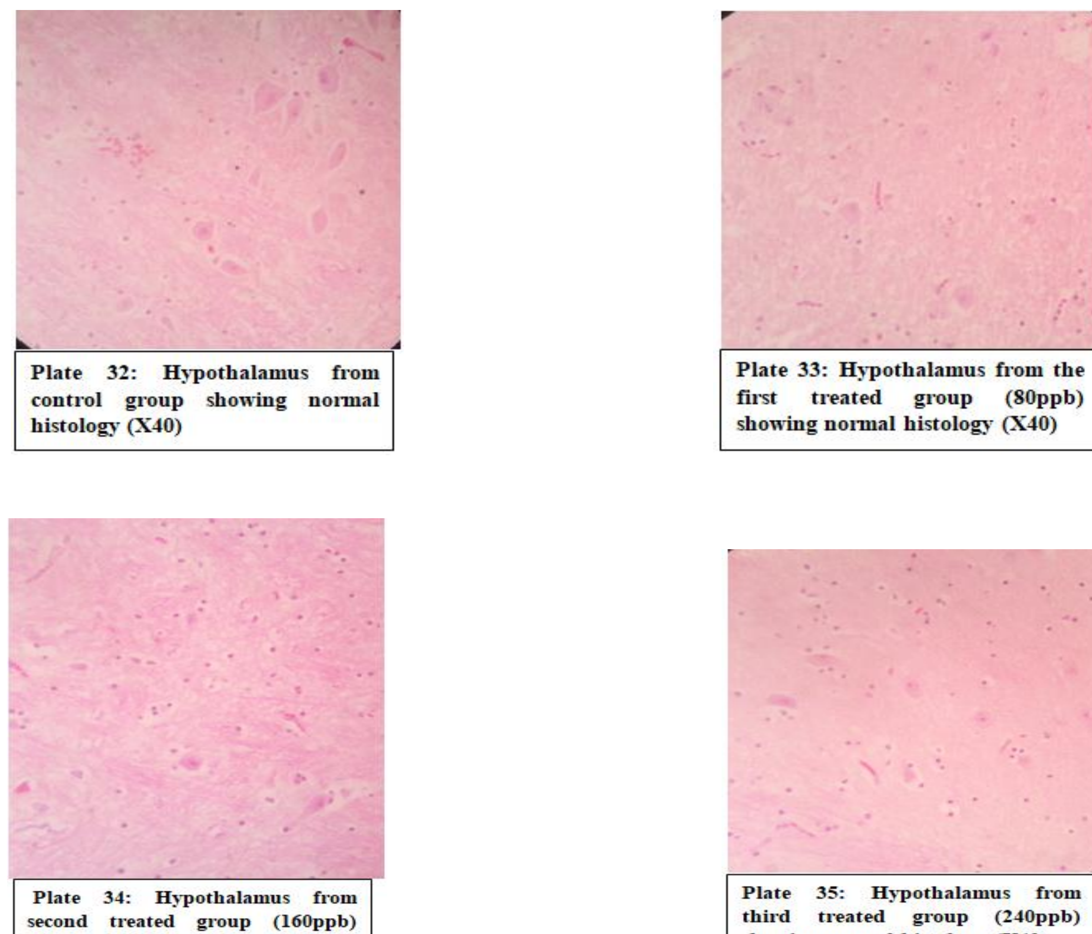


Plate 32: Hypothalamus from control group showing normal histology (X40)

Plate 33: Hypothalamus from the first treated group (80ppb) showing normal histology (X40)

Plate 34: Hypothalamus from second treated group (160ppb) showing normal histology (X40)

Plate 35: Hypothalamus from third treated group (240ppb) showing normal histology (X40)

Plate 28 to 35: Histopathological changes in the pituitary and hypothalamus

The results of this study show varying histopathological changes on the testes, epididymis and the liver tissue of AFB_1 treated male pigs. However, from the histological sections, the pituitary gland and the hypothalamus appeared to have normal structure as shown in plates 28 to 35.

CHAPTER FIVE

DISCUSSION

5.1 Body Weight and Testicular Volume

Administration of 80 ppb, 160 ppb and 240 ppb aflatoxin B_1 levels for 60 days did not have a significant effect on body weight. This observation may imply that probably, the three levels of aflatoxin B_1 administered to the pigs did not significantly affect the efficiency of feed conversion thus the rate of increase in body weight was not significantly affected. However, administration of 160 ppb and 240 ppb AFB_1 levels caused testicular degeneration as evidenced by the marked reduction in testicular volume in the second and third AFB_1-treated groups respectively. These findings are in agreement with those of Piskac *et al*. (1982) who reported that prolonged administration of aflatoxin B_1 to male rats and pigs resulted in degeneration of testicular tissues. Mohammed *et al*. (2014) also observed that contamination of human food with AFB_1 causes testicular degeneration. AFB_1 in this study brought about morphological regressive changes in the testicular tissue while the Leydig cells were seriously impaired. Aflatoxin is a known immunosuppressant (Dhanasekaran *et al*., 2011). Reduced immunity in the experimental pigs could have lead to the marked testicular degeneration in pigs fed higher aflatoxin levels (240 ppb).

5.2 Haematological Indices

The different aflatoxin B1 levels caused a significant reduction in WBC count, RBC, Hb, MCHC, lymphocytes, and monocyte count in the pigs in a dose-related manner. The levels of MCV and MCH increased significantly in the AFB_1 challenged groups. Aflatoxin is a known immunosuppressant due to its reactivity with T-cells, decrease in Vitamin K activities as well as a decrease in phagocytic activity in macrophages

(Dhanasekaran *et al*., 2011) and can therefore cause lymphopenia. Aflatoxins have also been reported to cause anaemia in animals (Dhanasekaran *et al*., 2011). Decrease in the RBC count have been attributed to retardation or disturbance in erythropoiesis in bone marrow (Candlish *et al*., 1986) as well as faster rate of destruction of peripheral red blood cells in the spleen. Aflatoxin has been reported to hamper RBC formation (Candlish *et al*., 1988). This may explain the elevated mean corpuscular haemoglobin in the aflatoxin-treated pigs as a result of macrocytic anaemia. Increased mean corpuscular volume may be attributed to destruction of hepatocytes since AFB_1 has been reported to induce apoptosis of hepatocytes. Aflatoxicosis has also been shown to cause lymphocytopenia and monocytopenia (Donmez *et al*. (2012). This implies that the levels of lymphocytes and monocytes in the blood was greatly reduced. The findings are in agreement with those of Elham & Mona (2004) who reported that AFB_1 brought about reduction in WBCs, RBCs, Hb, and MCHC but significantly increased the levels of MCV and MCH in male rats. The findings of this study are also in agreement with those of Donmez *et al*. (2012) who reported that AFB_1 caused a reduction in erythrocyte, leukocyte count, hemoglobin and hematocrit levels in rams fed 250 µg/day of aflatoxin B_1. Abdel-Wahhab *et al.* (2002) had earlier made similar observations that AFB_1 treatment significantly reduced blood haemoglobin, erythrocytes and leukocytes in male rats. The AFB_1 levels administered in this study had no significant effect on platelet count. This finding is in agreement with that of Sabourin *et al.* (2006) who reported that different AFB_1 doses did not affect platelet count in female mice. However, the finding in this study is not in agreement with that of Sun *et al*. (2015) who reported that aflatoxins decreased the number of platelet count in piglets fed 20µg/kg in feed. Probably the aflatoxin B_1 levels administered to the animals in the present study had no significant effect on platelet activity. The mechanisms through

which aflatoxin caused reduction in white blood cell count, red blood cell count and the haemoglobin have not been clearly understood.

5.3 Plasma Hormone Levels

In this study, the plasma levels of luteinizing hormone, testosterone and estradiol of the aflatoxin B_1 challenged groups were significantly reduced in a dose-related manner. Reduction in the levels of testosterone and estradiol may be attributed to reduced number of Leydig cells as well as degeneration of Sertoli cells in the testes of the AFB_1 treated pigs. One of the most common mechanisms suggested for the action of AFB_1 is the binding of DNA to form complexes and inhibit nucleic acid synthesis (Bbosa *et al*., 2013). This mode of action may explain the direct effect of aflatoxin B_1 on Leydig cells and Sertoli cells in the testes, and consequently the reduction of the sex steroid hormones namely testosterone and estradiol. The reduced luteinizing hormone levels can be explained by hypophysotoxic effects of aflatoxin especially on adenohypophysis (Hasanzadeh *et al*., 2011).The regulation of FSH is similar to that of LH except for the specific inhibitory effect of inhibin B on the production and secretion of FSH. Due to the effect of aflatoxin B_I on sertoli cells inhibin B level was reduced thus lowering its inhibitory effect on FSH. This mechanism would naturally lead to elevated levels of FSH but due to hypophysotoxic effects of aflatoxin FSH level was not significantly affected by the treatment (Mabeck *et al.,* 2005). The findings in this study are in agreement with those of Ewuola *et al*. (2014) who reported that goats fed higher AFB_1 levels had significantly lower testosterone levels compared to the control group. Clarke *et al*. (1987) had earlier reported similar results in male chicken fed on varying levels of dietary aflatoxin. Their results showed reduced plasma testosterone levels, as well as reduced levels of plasma luteinizing hormone in the aflatoxin treated groups compared

to the control group. Plasma testosterone levels have been shown to reduce in white leghorn male chicken and in birds fed varying levels of AFB_1 contaminated feed as described by Bbosa *et al*. (2013). The results of this study are also in agreement with the findings of Hasanzadeh *et al*. (2011) who reported that aflatoxin B_1 causes reduction in the concentration of testosterone as well as 17β-estradiol in aflatoxin treated male rats. The AFB_1 levels administered to the pigs did not have any significant effect on FSH concentration. However, luteinizing hormone was significantly reduced in the AFB_1 treated pigs in a dose-related manner. These findings differ partly from the findings of Hasanzadeh *et al*. (2011) who reported a significant increase in the levels of FSH, but are in agreement regarding the reduced level of LH in aflatoxin fed male rats.

5.4 Histopathological Changes Caused by aflatoxin B_1

This study showed varying histopathological changes on the testes, epididymis, liver, pituitary gland and hypothalamus which ranged from moderate to severe in the 160 ppb and 240 ppb AFB_1-treated groups respectively. Histopathological changes in the testes of the pigs challenged with AFB_1 included progressive decrease of spermatogenesis cells and progressive reduction of Leydig cells and Sertoli cells in a dose-dependent manner. Findings in this study are in agreement with those of Murad *et al*. (2015) who observed similar effects on the reproductive system of Albino male rats where the number of Leydig cells, Sertoli cells and spermatogenesis cells were significantly reduced with increased AFB_1 concentration. Other observations of the testes in this study were; progressive increase in peritubular oedema, necrosis of seminiferous tubules and atrophy of the seminiferous tubules characterized by thickened basement membrane. Although the first treated group (80 ppb) showed mild testicular changes,

these changes were so marked in the third treated group (240 ppb). These findings are in agreement with those of Lakkawar *et al*. (2004) who observed similar testicular changes including oedematous interstitial tissue, atrophy of spermatogenic epithelium and tissue oedema as well as reduced number of mature spermatozoa in young rabbits fed AFB_1 levels of 0.5 ppm/kg of feed for 50 days.

Similar testicular changes were observed by Elham & Mona (2004) in male rats fed 7.5/mg/200 grams body weight of AF for three weeks. They observed degeneration of the lining of the epithelium of seminiferous tubules and congestion of testicular blood vessels with intertubular oedema in the treated group. These observations were also previously reported by Ahmed *et al*. (2012) when they noted pathological alterations in the testes of aflatoxicated rabbits including degeneration of spermatogonial cells lining seminiferous tubules, peritubular oedema associated with marked atrophy of seminiferous tubules and disturbed process of spermiogenesis. Ortatatli *et al.* (2002) had earlier made similar observations including absence of spermatogenesis in the testes, degeneration and desquamation in the epithelium and decrease in the size and thickness of the germinative layer of the seminiferous tubules in adult roosters.

Microscopic examination on the epididymis in this study showed epithelia hyperplasia and cytoplasmic vacuolation in all pigs treated with aflatoxin B_1 different from the control pigs. These findings agree with that of Ahmed *et al*. (2012) who observed hyperplasia of the epididymal epithelial cells as well as cytoplasmic vacuolation in male rabbits treated with 250, 500 and 1000 ppb aflatoxin for 60 days. These epithelial cells of the epididymis are already terminally differentiated cells and usually, they do not divide unless induced to mitosis. Since the epididymis of all the AFB_1-treated pigs in this study showed epithelial hyperplasia, it may imply that aflatoxin B_1 is a potent

mitogenic agent, and a potentially carcinogenic agent in respect to epididymis (Ahmed *et al*., 2012).

Histopathological changes in the liver tissue in this study included; marked bile duct proliferation predominantly in the 160 ppb and 240 ppb aflatoxin B_1-treated pigs; dilatation of the central vein; mononuclear cell infiltration, steatosis, fibrosis and marked congestion of the parenchyma. The liver is the principal target organ for toxic effects of aflatoxin B_1. According to Dhanasekaran *et al*. (2011) AFB_1 causes a decrease lipid metabolism in the liver as well as impair the transport of synthesized lipids out of the liver. This leads to fatty liver as well as cytoplasmic vacuolation, with many of these vacuoles containing fat. Aflatoxin B_1 also induces apoptosis and morphological changes in hepatocytes (Harutyunyan, 2015), a mechanism that may account for a number of histopathological changes observed in the liver. Cholesterol is a requirement for formation of steroid hormones. This is inclusive of sex steroid hormones testosterone and estradiol (Hames & Hooper, 2005). The liver is the major site of synthesis and regulation of cholesterol, accounting for 80% *denovo* synthesis. The direct toxic effects of AFB_1 on the liver may affect hepatocyte functioning and may explain the reduced levels of sex steroid hormones testosterone and estradiol in the AFB_1-treated pigs. These findings are in agreement with those of Elham & Mona (2004) who made similar observations on the liver of rats fed on 7.5mg/200grams body weight of aflatoxin for 3 weeks. They observed lymphocytic cellular aggregation, inflammatory cellular infiltration of portal area mostly lymphocytes, congested portal vessels, dilatation of the central vein as well as presence of small newly formed bile ducts. Bbosa *et al*. (2013) report similar AFB_1 related changes on the liver including congestion of liver parenchyma, cytoplasmic vacuolation or fatty change of hepatocytes, newly formed bile ducts as well as mononuclear cell infiltration in broiler

chicks. Marai & Asker (2008) reported on related histopathologic changes in the liver tissue of calves where daily aflatoxin doses as low as 0.04mg/kg body weight within a period of 6 days resulted in bile duct proliferation, perivascular oedema, and fibroblastic infiltration. The findings are also in agreement with those of Lakkawar *et al*. (2004) who reported fatty change, coagulative necrosis around the central veins, mononuclear cellular infiltrations, portal fibrosis and bile duct proliferation in rabbits. The control group in this study showed normal histology of the liver with no significant changes. The mechanism of action of aflatoxin B1 on these tissues is not clearly understood, although the observed histopathological changes in the liver, testis and epididymis aided in answering the physiological question which was the main objective of this study.

The histology sections of the pituitary gland and of the hypothalamus appeared to have normal structures under light microscope. The three cell types of the adenohypophysis in the pituitary gland namely the acidophils, basophils and chromophobes could be distinguished in the examined histology sections.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the findings of the present study showed that oral administration of aflatoxin B_1 levels to mature male pigs (*Sus scrofa domesticus*):

1. AFB_1 did not have any significant effect on the animals' body weight.
2. AFB_1 caused a significant reduction in testicular volume in a dose-related manner.
3. AFB_1 caused a decrease in WBC, RBC, Hb, and MCHC. The toxin significantly increased MCH and MCV. AFB_1 had no significant effect on platelet count.
4. AFB_1 significantly decreased the levels of LH, testosterone and estradiol but had no significant effect on FSH levels.
5. AFB_1 caused histopathological changes in the testes, epididymis and liver.
6. AFB_1 did not cause any notable histological changes in the pituitary gland and the hypothalamus.

6.2 Recommendations

From the findings of the present study, it is recommended that:

1. Great care should be taken to ensure aflatoxin-free feeds due to the close link between aflatoxin-contaminated feeds and animal reproduction.
2. Proper testing of aflatoxin B_1 levels in swine feeds be carried out to ensure aflatoxin-free feeds for animal consumption.
3. Further research be undertaken to investigate the effects of AFB_1 on HPG axis in female pigs.
4. More research be carried out on the effects of AFB_1 on HPG axis before puberty, during puberty or later stages of reproduction in pigs.

REFERENCES

Abdel-Wahhab, M., Nada, A. & Khalil, F. (2002). Physiological and toxicological responses in rats fed aflatoxin contaminated diet with or without sorbent materials. *Journal of Animal Feed Science and Toxicology, 97(*314), 209-219.

Ahmed, K., Mahady, M., Badawy, S., Ahmed, Y., & Aly, M. (2012). Pathological consequences of aflatoxins in male rabbit: Cellular, Genetic and Oxidative Damage. *Globa Veterinaria 9*(1), 42-52. Retrived from idosi.org/gv/GV9(1)12/7.pdf

Ajani, J., Chakravarthy, D. V. S., Tanuja, P., & Pasha, K. V. (2014). Aflatoxins. *Indian Journal of Advances in Chemical Science*, *3*, 49–60.

Akbarsha, M., Kunnathodi, F., & Alshatwi, A. (2008). A comprehensive review of male reproductive toxic effects of aflatoxin. *Aflatoxins-Biochemisty and Molecular Biology* pp.177-202

American Heritage Medical Dictionary (2007). Houghton mifflin company. Retrieved from http://medical-dictionary.thefreedictionary.com/aflatoxicosis

Bagheri, T., Moshtaghi, H., & Boniadian, M. (2014). Aflatoxin B 1 in feed stuffs of dairy farms in Shahrekord, Iran. *Journal of Novel Applied Sciences, 3(*11), 1312–1316.

Bakırdere, S., Bora, S., Bakırdere, E., Aydın, F., Arslan, Y., Komesli, O. & Yıldırım, E. (2012). Aflatoxin species: their health effects and determination methods in different foodstuffs. *Central European Journal of Chemistry*, *10*(3), 675–685. http://doi.org/10.2478/s11532-012-0009-2

Bbosa, G., Kitya, D., & Lubega, A. (2013). Review of the biological and health effects of aflatoxins on body organs and body systems. *Aflatoxin:Recent Advances and Future Prospects*, pp 240 – 265. Retrived from http://dx.doi.org/10.5772/51201

Candlish, A., Stimson, W. & Smith, J. (1986). A monoclonal antibody to ochratoxin. Toxicon, 27 (8), 887-897

Candlish, A., Stimson, W. & Smith, J. (1988). Determination of ochratoxin A. by monoclonal antibody-based enzyme immunoassay. *Journal -Association of official Analytical Chemistry, 71:* 961.

Clarke, R., Doerr, J. & Ottinger, M. (1987). Age-related changes in testicular development and reproductive endocrinology associated with aflatoxicosis in the male chicken. *Biology of Reproduction*, *36*(1), 117–124. http://doi.org/10.1095/biolreprod36.1.117

Cooper, G. T. (2009) Cooper, G. T. (2009). Human Reproduction Update. World Health Organization reference values for human semen characteristics. Vol.16, No.3 pp. 231–245.

Crowley, F., Filicori, M., Spratt, D. & Santoro, F. (1985). The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Rec Proc Horm Res 41:473-531.

Crowley, F., Whitcomb, W., Jameson, L., Weiss, J., Finkelstein, J. & O'Dea, L. (1991). Neuroendocrine control of human reproduction in the male. Recent Prog Hormone Res 47:27-67.

Dell, R.B., Holleran, S., & Ramakrishnan R. (2002). Sample size determination. *Institute for Laboratory Animal Research Journal 43*(4); 207-13. Retrived from www.ncbr.nlm.nih.gov/pubmed/ 1239/396

Dhanasekaran, D., Shanmugapriya, S., Thajuddin, N. & Panneerselvam, A. (2011). Aflatoxins and aflatoxicosis in humans and animals. Retrived from cdn.intechopen.com/pdfs

Donmez, N., Donmez, H., Keskin, E., and Kisadere I. (2012). Effects of aflatoxin on some haematological parameters and protective effectiveness of esterified glucomanna in Merion Rams. *Scientific World Journal*. Doi. 10.1100/2012/342468.

El-Azab, S. M., Abdelhamid, A. M., Shalaby, H. A., Mehrim, A. I. & Ibrahim, A. H. (2010). A study of Aflatoxin B_1 as a risk factor that impairs the reproductive performance in females – Egypt. *Toxicological & Environmental Chemistry*, *92*(2), 383–389. http://doi.org/10.1080/02772240902927510

Elham A. & Mona F. (2004). Ameliorative effect of vitamin E against the toxicity of aflatoxin B1 on rats with special reference to its effect on male fertility. *1rst Ann. Confr.,FVN., Moshtohor.* University of Benha Branch. pp 189-214

Ewuola, E.O., Jimoh, O.A., Bello, A.D., Bolarinwa, A.O. (2014). Testicular biochemicals, sperm reserves and daily sperm production of West African dwarf bucks fed varied levels of dietary aflatoxin. *Animal Reproduction Science. 148*(3-4), 182-187.

Faridha, A., Faisal, K., & Ma, A. (2006). Duration-dependent histopathological and histometric changes in the testis of aflatoxin b1-treated mice. *Journal of Endocrinol Reproduction, 10*(2), 117–133.

Feddern, V., Dors, C., Tavernari, D., Mazzuco, H., Krabbe, E. & Scheuermann, N. (2013). Aflatoxins Importance on Animal Nutrition. Retrived from http://dx.doi.org/10.5772/51952

Groesbeck, C., Goodband, R., DeRouchey, J., Tokach, M., Dritz, S., Nelssen, J., Lawrence, K., & Young, M. (2002). Using Heart Girth to Determine Weight in Finishing Pigs. Retrieved from https:www.asi.k-state.edu/doc/swine

Harvey, R.B., Kubena, L.F., Elissalde, M.H., Corrier, D.E., Huff, W.E., Rottinghaus, G.E. & Clement, B.A. (1991). Cocontamination of Swine diets by aflatoxin and diacetoxyscirpenol. *Vet Diagn Invest 3*, pg 155-160.

Harutyunyan, T. (2015). Apoptosis-inducing effect of AFB_1, Ochratoxin A and Zearalenone combination in rat hepatocytes.

Hames, D. & Hooper, N. (2005). Biochemistry. 3rd edition. Taylor & Francis Group. United Kingdom. Pages 357-358.

Hasanzadeh, S. & Amani, S. (2013). Aflatoxin B1 effects on ovarian follicular growth and atresia in the rat. *Journal of Comparative Clinical Pathology*, *22*(4), 563–572. http://doi.org/10.1007/s00580-012-1446-1

Hasanzadeh, S., Hosseini, E., & Rezazadeh, L. (2011). Effects of aflatoxin B 1 on profiles of gonadotropic (FSH and LH), steroid (testosterone and 17β-estradiol) and prolactin hormones in adult male rat. *Iranian Journal of Veterinary Research*, *12*(4), 332–336.

Hasanzadeh, S., & Rezazadeh, L. (2013). Effects of aflatoxin B1 on the growth processes of spermatogenic cell series in adult male rats. *Journal of Comparative Clinical Pathology*, *22*(4), 555–562. http://doi.org/10.1007/s00580-012-1445-2

Jensen, TK., Andersson, AM., Jorgensen, N., Andersen, AG., Carlsen, E., Petersen, JH., & Skakkebaek, NE. (2004). Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. PubMed 82 (4): 863-70.

Johnson, P.J. (1984). Sex hormones and the liver. Clinical Science (66) 369-376. Retrieved from http://www.clinsci.org/content/ppclinsci/66/4/369.full.pdf

Kanora, A., & Maes, D. (2009). The role of mycotoxins in pig reproduction: A review. *Veterinarni Medicina*, *54*(12), 565–576.

Ketterer, P.J., Blaney, B.J., Moore, C.J., McInnes, I.S. & Cook, P.W. (1982). Field cases of aflatoxicosis in pigs. *Australian Veterinary Journal 59* (4), 113-118. Doi: 10.1111/j.1751-0813.1982.tb02743.x

Lakkawar, W., Chattopadhyay, K. & Johri, S. (2004). Experimental aflatxoin B1 toxicosis in young rabbits: A clinical and patho-anathomical study. *Slov Vet Res, 41*(2)*,* 73-81.

Lin, C., Huang, J. & Chen, K. (2009). Measurement of testicular volume in smaller testes: How accurate is the conventional orchidometer? *Journal of Andorology 30*(6).

Mabeck, L., Jensen, M., Toft, G., Thulstrup, M., Andersson, M., Jensen, T., Giwercman, A., Olsen, J. & Bonde, J.P. (2005). Fecundability according to male serum inhibin B—a prospective study among first pregnancy planners. *Human Reproduction 20*(10),2909–2915.

MacDonald,A., Stewart, A. & Farquhar, C.(2013). Body mass index in relation to semen quality and reproductive hormones in New Zealand men: a cross-sectional study in fertility clinics.

Marai, M. & Asker, A. (2008). Aflatoxins in rabbit production: Hazards and control. *Tropical and Subtropical Agroecosystems, 8* (1), 1-28.

McLean, M., Dutton, M.F. (1995). Cellular interactions and metabolism of aflatoxin an update. *Pharmacology and thearapeutics, 65*, 163-192.

Mohammed, M., Ameen, M., Mohammed, O., AlMaghraby, O., Aziz, H., Ahmed, S. & Alsammani, M. (2014). The Effect of aflatoxins on male reproduction. *Medical Archives*, *68*(4), 272. http://doi.org/10.5455/medarh.2014.68.272-275

Murad, F., Ahmed S., & Abead S. (2015). Toxicity effect of aflatoxin B1 on reproductive system of albino male rats. *Pakistan Journal of Biological Sciences, 2015. Asian Network for Scientific Information.18*(3),107-114.

Nitsche et al. (2014). Testosterone changes in patients with liver cirrhosis before and after orthotopic liver transplantation and its correlation with meld. *Arquivos de gastroenterologia*, *51*(1), 59-63. https://dx.doi.org/10.1590/s0004-28032014000100012

Ortatatli, M., Ciftci, K., Tuzcu, M. & Kaya, A. (2002). The effects of aflatoxin on the reproductive system of roosters. *Research in Veterinary Science 2002,* (72)*,* 29-36. Harcourt Publishers limited. Doi: 10.1053.rvsc.2001.0516. Retrived from http://www.idealibrary.com

Osweiler, G.D. (2014). Aflatoxicosis. *Merck veterinary manual*. Retrieved from www. merckvetmanual.com/mvm/toxicology/mycotoxicoses/aflatoxicosis.html

Pierre, S. (2012). ISARA -Lyon. Structure of aflatoxins. Retireved on 21st May 2016 from http://www.mf.uni-mb.si/mf/instituti/IPweb2012/html/S_Pierre_Aflatoxins%20-.pdf

Piskac, A., Drábek, J., Halouzka, R. & Groch, L. (1982). The effect of long term administration of aflatoxins on the health status of male rats and pigs with respect to morphological changes in the testes. *Veterinární Medicína, 27*(2)*,* 101-111. ISSN 0375-8427

Sabourin, J., Price A., Casbohn, L., Perry, R., Tuttle, S., Rogers, V., Rowell, S., Estep, E. & Sabourin, L. (2006). Evalutaion of acute immunotoxicity of aerosolized aflatoxin B_1in female C57BL/6N mice. *Journal Immunotoxicol, 3*(1)*,*11-20. doi:10.1080/15476910500468635

Saladin (2009). Anatomy and physiology 5th Edition (pp 670-672). Retrieved from http://www.cram.com/flashcards/endocrine-systemhormones-from-chapter-17-saladin-937704

Sayo, E. (2015). Aflatoxicosis. Retrieved from clinicalmed.uonbi.ac.ke

Scanlon & Sanders (2007). Essentials of Anatomy and Physiology (pp 224-229). Retrieved from https://www.amazon.com/Essentials-Anatomy-Physiology-Valerie Scanlon/dp/0803622562

Sun, Y., Park, I., Guo, I., Weaver, A. & Sung Woo K. (2015). Impacts of low level aflatoxin in feed and the use of modified yeast cell wall extract on growth and health of nursery pigs. *Journal Animal Nutrition*, *1*(3), 177-183.

Tony, M. (2015). The hypothalamo-pituitary-gonadal axis. *Journal of Endocrinolog,226* (2): T41-T54.

Turner, P. C., Sylla A., Gong Y.Y., Diallo M.S., Sutcliffe A. E., Hall, A.JWild C.P (2005). Reduction in exposure to carcinogenic aflatoxins by postharvest intervention measures in West Africa: A community based intervention study. *The Lancet, 365,* 1950-1956.

United States Department of Agriculture (USDA) Aflatoxin handbook (March 2002). Retrieved from centur.vri.cz/news/prilohy/pril351.pdf

Vance D. & Vance J. (2002). Biolchemistry of lipids, lipoproteins and membranes (4th edition). Elsevier Science B.V.

Verma, R.J., & Raval, P.J. (1992). Alterations in erythrocytes during induced chronic aflatoxicosis in rabbits. *Bulletin of Environmental Contamination and Toxicology, 49* (6), 861-865

Wild, C. P., & Gong, Y. Y. (2009). Mycotoxins and human disease: A largely ignored global health issue. *Carcinogenesis*, *31*(1), 71–82. http://doi.org/10.1093/carcin/bgp264

https://www.researchgate.net/figure/230245326_fig1_Fig-1-The-hypothalamic-pituitary-testicular - axis

APPENDICES

Appendix I: Definition of Operational Terms

Aflatoxin: The name aflatoxin has been derived from the combination of three words; "a" for the *Aspergillus* genus, "fla" for the species *flavus*, and toxin meaning poison. It is a highly toxic compound produced as secondary metabolite produced by fungi belonging to several *Aspergillus* species, mainly *A. flavus* and *A. parasiticus* (Bakırdere *et al*., 2012).

Aflatoxicosis: This is poisoning caused by consumption of substances or foods contaminated with aflatoxin. (American Heritage Medical dictionary)

ELISA: This is an abbreviation which stands for Enzyme-linked immunosorbent assay. It is an immunological technique that uses an enzyme linked to an antibody or antigen as a marker for the detection of a particular protein, especially an antigen or an antibody, from a sample such as blood sample.

Hypothalamic-pituitary-gonadal (HPG) axis: The hypothalamic-pituitary-gonadal (HPG) axis involves the hypothalamus, the pituitary gland and the gonads which are endocrine glands that function as a single entity in the development and regulation of a number of body systems. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which targets the anterior pituitary gland. The anterior portion of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH) , and the male gonads produce testosterone (Childress 2010).

Gonadotropin releasing hormone (GnRH): This is a hypothalamic neuropeptide that regulates secretion of both luteinizing hormone and follicle-stimulating hormone by the anterior portion of the pituitary gland.

Luteinizing hormone (LH): This is a gonadotropic hormone secreted by the anterior pituitary gland in response to gonadotropin-releasing hormone secreted in the hypothalamus. In men, LH stimulates the interstitial cells of the testes to secrete testosterone, thus it is also called interstitial cell stimulating hormone (Scanlon & Sanders 2007).

Follicle stimulating hormone (FSH): This is a gonadotropic hormone secreted in the anterior pituitary gland. Its secretion is stimulated by the hypothalamus which produced gonadotropin-releasing hormone (GnRH). In men, FSH initiates sperm production within the testes. FSH secretion is decreased by inhibin B, a hormone produced by the testes in males (Scanlon & Sanders 2007).

Testosterone: This is a hormone secreted by the testes when stimulated by luteinizing hormone. It is responsible for the development of the male reproductive system as well as the masculine stature in men and sex drive. Testosterone sustains sperm production and sexual instinct during the reproductive stage in males (Saladin, 2009).

Estradiol (E2): This hormone is synthesized by Leydig cells in response to luteinizing hormone (LH) and by Sertoli cells in response to follicle-stimulating hormone (FSH) in male mammals. (Scanlon & Sanders 2007)

Appendix II: Preparation of Aflatoxin B_1 Working Solution

Aflatoxin dosage was prepared in the ratio of 1:2:3 with 80 ppb for the 1st treatment group, 160 ppb for the 2nd treatment group and the 240 ppb for the 3rd treatment group. 84.6 mg of aflatoxin was weighed on analytical balance and then transferred to a conical flask. The aflatoxin was then dissolved in 8.64 ml of dichloromethane and methanol in a ratio of 1:1 to give a clear colourless stock solution of the aflatoxin. 10 mg/ml of dichloromethane and methanol was used; therefore 8.64 ml of dichloromethane/methanol was used to dissolve the aflatoxin. This means that 4.32 ml of dichloromethane and 4.32 ml of methanol was used to give the ratio of 1:1.

Administration of aflatoxin was done in 3 doses, which are 80 ppb, 160 ppb and 240 ppb.

Three pigs in a day consumes

3 x 80 = 240 ppb

3 x 160= 480 ppb

3 x 240 = 720 ppb

Hence 9 pigs would consume 240+480+720 = 1440 ppb in a day.

Therefore in 60 days the pigs consume 1440 x 60 = 86400 ppb

86400 ppb = 86.4 mg/l

1 mg/l = 1000 ppb

1st dosage: 80ppb x 3 pigs x 60 days = 14,400 ppb = 14.4 mg/l

14400/1000= 14.4

Thus 14.4 litres of distilled water dissolved 14.4 mg of aflatoxin.

2nd dosage: 160 ppb x 3 x 60 = 28800ppb = 28.8 mg/l

28800/1000 = 28.8 litres of distilled water dissolved 28.8 mg of aflatoxin.

3rd dosage: 240 ppb x 3 x 60 days = 43200 ppb = 43.2 mg/l

43200/1000 = 43.2 litres of distilled water dissolved 43.2 mg of aflatoxin.

Total volume = 86.4 litres

Concentrating the solution: 86.4 / 4 = 21.6 litres

21.6 litres in 86.4 mg of aflatoxin

Implying that the dosage was given as

80 ppb/4 = 20 ppb was measured

160 ppb / 4 = 40 ppb was measured

240 ppb/ 4 = 60 ppb was measured

Each dosage was topped up with distilled water to make a final volume of 75 ml of aflatoxin in each dispensing bottle.

Dispensing bottles required = 180 x 3 = 540

Total volume in all the 540 vials was 540 x 75 = 40500 mls = 40.5 L

Appendix III: Spectrophotometric Results for Follicle Stimulating Hormone

1	2	3	4	5	6	7	8	9	10	11	12
standard 2.864	standard 2.737	standard 1.748	standard 2.176	standard 1.052	standard 0.937	standard 0.465	standard 0.489	standard 0.335	standard 0.310	Blank control 0.092	Sample 1 0.768
2 0.754	3 0.722	4 0.762	5 0.637	6 0.729	7 1.791	8 0.626	9 0.579	10 0.582	11 0.669	12 0.609	13 0.780
14 0.639	15 0.740	16 1.785	17 1.440	18 0.662	19 0.958	20 0.649	21 0.522	22 0.626	23 0.566	24 1.058	25 1.622
26 0.848	27 0.858	28 0.802	29 0.867	30 0.520	31 1.546	32 0.550	33 0.606	34 1.776	35 0.643	36 0.640	37 0.596
38 0.815	39 0.552	40 0.942	41 0.528	42 0.550	43 1.690	44 0.462	45 0.459	46 0.826	47 0.426	48 0.378	49 0.713
50 0.544	51 0.563	52 1.661	53 0.594	54 0.452	55 0.582	56 0.433	57 0.426	58 0.595	59 0.379	60 0.533	61 1.510
62 0.518	63 0.514	64 0.569	65 0.696	66 0.628	67 0.498	68 0.656	69 0.385	70 0.381	71 0.560	72 0.623	73 0.410
74 0.901	75 0.566	76 0.542	77 0.477	78 0.403	79 0.486	80 0.460	81 0.419	82 0.285	83 0.426	84 0.496	73 0.402

Appendix IV: Spectrophotometric Results for Luteinizing Hormone (LH)

1 standard 0.737	2 standard 0.805	3 standard 0.590	4 standard 0.641	5 standard 0.315	6 standard 0.327	7 standard 0.195	8 standard 0.168	9 standard 0.119	10 standard 0.027	11 Blank 0.014	12 Sample 1 0.143
2 0.136	3 0.119	4 0.125	5 0.081	6 0.152	7 0.405	8 0.122	9 0.129	10 0.155	11 0.092	12 0.097	13 0.106
14 0.124	15 0.158	16 0.304	17 0.405	18 0.102	19 0.175	20 0.097	21 0.120	22 0.124	23 0.108	24 0.359	25 0.457
26 0.237	27 0.118	28 0.181	29 0.174	30 0.094	31 0.375	32 0.123	33 0.139	34 0.634	35 0.146	36 0.098	37 0.120
38 0.174	39 0.111	40 0.195	41 0.121	42 0.137	43 0.468	44 0.117	45 0.128	46 0.229	47 0.098	48 0.102	49 0.131
50 0.099	51 0.119	52 0.368	53 0.099	54 0.114	55 0.120	56 0.109	57 0.094	58 0.229	59 0.070	60 0.138	61 0.425
62 0.127	63 0.104	64 0.159	65 0.139	66 0.275	67 0.145	68 0.114	69 0.120	70 0.120	71 0.139	72 0.087	73 0.092
74 0.176	75 0.123	76 0.134	77 0.189	78 0.109	79 0.109	80 0.137	81 0.141	82 0.133	83 0.109	84 0.162	49 0.130

Appendix V: Spectrophotometric Results for Testosterone Hormone

1 standard 2.117	2 standard 1.863	3 standard 1.859	4 standard 1.712	5 standard 1.229	6 standard 1.371	7 standard 0.903	8 standard 0.748	9 standard 0.343	10 standard 0.609	11 Blank 0.095	12 Sample 1 0.667
2 0.581	3 0.563	4 0.506	5 0.468	6 0.547	7 1.344	8 0.691	9 0.647	10 0.744	11 0.747	12 0.663	13 0.751
14 0.500	15 0.496	16 1.217	17 0.876	18 0.508	19 0.858	20 0.571	21 0.601	22 0.587	23 0.787	24 0.869	25 1.477
26 0.712	27 0.545	28 0.622	29 0.743	30 0.539	31 1.213	32 0.526	33 0.693	34 1.407	35 0.796	36 0.618	37 0.708
38 0.654	39 0.458	40 0.770	41 0.467	42 0.507	43 1.553	44 0.497	45 0.499	46 0.809	47 0.652	48 0.580	49 0.776
50 0.543	51 0.493	52 1.360	53 0.556	54 0.414	55 0.612	56 0.426	57 0.462	58 0.966	59 0.561	60 0.557	61 1.348
62 0.448	63 0.456	64 0.500	65 0.627	66 0.463	67 0.533	68 0.561	69 0.649	70 0.661	71 0.579	72 0.437	73 0.618
74 0.765	75 0.501	76 0.548	77 0.651	78 0.490	79 0.464	80 0.573	81 0.589	82 0.614	83 0.663	84 0.602	64 0.538

Appendix VI: Spectrophotometric Results for Estradiol (E2)

1	2	3	4	5	6	7	8	9	10	11	12
standard 3.022	standard 2.901	standard 2.222	standard 2.323	standard 1.766	standard 1.801	standard 0.856	standard 0.895	standard 0.746	standard 0.664	Blank 0.087	Sample 1 1.181
2 1.143	3 0.946	4 0.927	5 0.944	6 0.758	7 1.837	8 0.923	9 0.832	10 1.104	11 1.003	12 0.736	13 1.140
14 0.874	15 0.927	16 1.717	17 1.373	18 0.764	19 1.166	20 0.851	21 0.820	22 0.997	23 0.994	24 1.253	25 2.001
26 1.268	27 0.932	28 1.004	29 1.116	30 0.834	31 1.753	32 0.916	33 0.949	34 1.954	35 1.006	36 0.840	37 0.945
38 1.194	39 0.833	40 1.219	41 0.827	42 0.848	43 1.904	44 0.802	45 0.822	46 1.067	47 0.830	48 0.736	49 1.250
50 1.058	51 0.143	52 1.942	53 0.692	54 0.679	55 1.417	56 0.746	57 0.799	58 1.658	59 0.914	60 1.031	61 2.007
62 0.998	63 0.135	64 0.910	65 1.027	66 0.847	67 0.817	68 1.350	69 0.941	70 1.000	71 1.318	72 0.956	73 0.897
74 1.394	75 0.142	76 0.932	77 0.937	78 0.817	79 0.752	80 0.796	81 1.351	82 0.951	83 0.936	84 1.111	

Appendix VII: Research Authorization

NATIONAL COMMISSION FOR SCIENCE, TECHNOLOGY AND INNOVATION

Telephone: +254-20-2213471,
2241349, 310571, 2219420
Fax: +254-20-318245, 318249
Email: secretary@nacosti.go.ke
Website: www.nacosti.go.ke
When replying please quote

9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/16/44726/9603**

Date: **15th April, 2016**

Mukumu Catherine Kivinya
Moi University
P.O Box 3900-30100
ELDORET.

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on ***"Effects of aflatoxin on the functions of the hypothalamic-pituitary-gonadal axis in male pigs,"*** I am pleased to inform you that you have been authorized to undertake research in **Kakamega and Nairobi Counties** for the period ending **13th April, 2017.**

You are advised to report to **the County Commissioners and the County Directors of Education, Kakamega and Nairobi Counties** before embarking on the research project.

On completion of the research, you are expected to submit **two hard copies and one soft copy in pdf** of the research report/thesis to our office.

DR. STEPHEN K. KIBIRU, PhD.
FOR: DIRECTOR-GENERAL/CEO

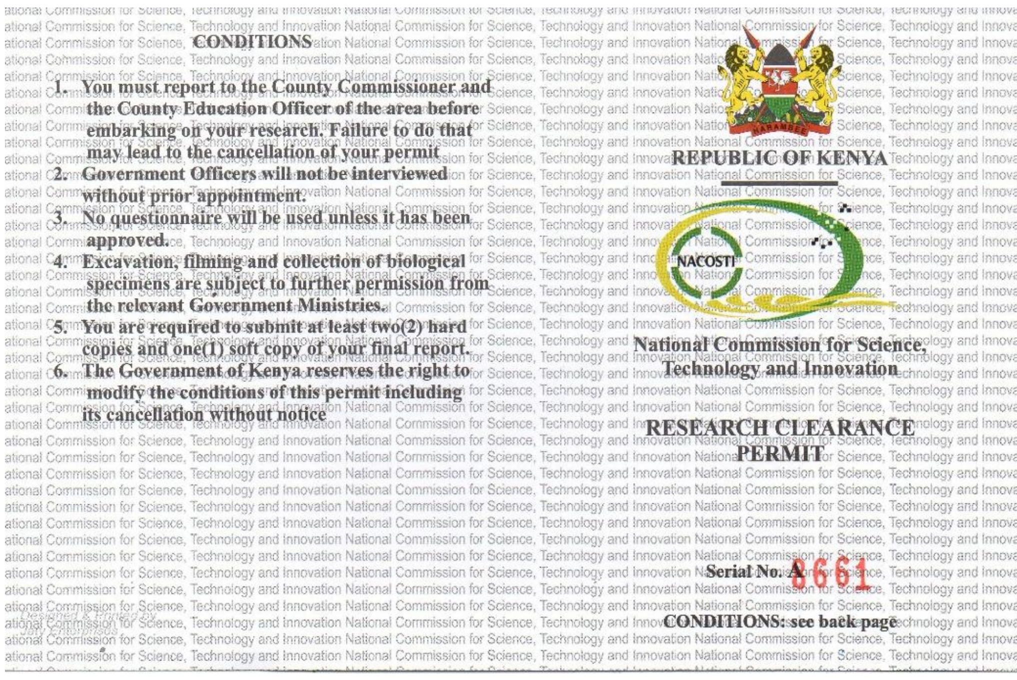
Copy to:

The County Commissioner
Kakamega County.

The County Director of Education
Kakamega County.

National Commission for Science, Technology and Innovation is ISO 9001: 2008 Certified

Appendix VIII: Research Permit



CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two(2) hard copies and one(1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice

REPUBLIC OF KENYA

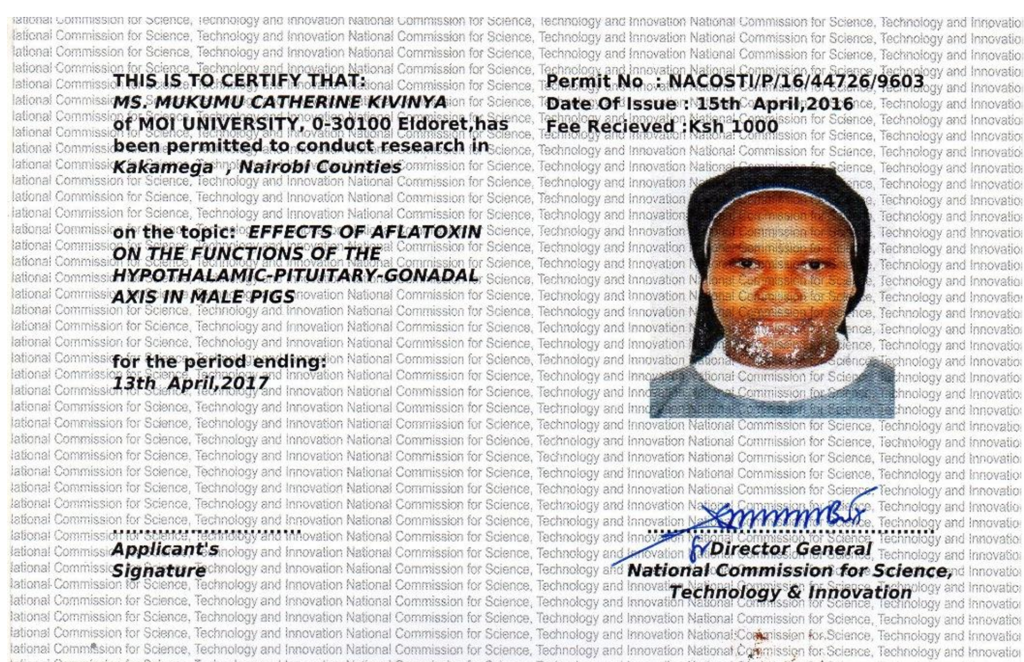
NACOSTI

National Commission for Science, Technology and Innovation

RESEARCH CLEARANCE PERMIT

Serial No. A 8661

CONDITIONS: see back page



THIS IS TO CERTIFY THAT:
MS. MUKUMU CATHERINE KIVINYA
of MOI UNIVERSITY, 0-30100 Eldoret, has been permitted to conduct research in *Kakamega , Nairobi Counties*

on the topic: *EFFECTS OF AFLATOXIN ON THE FUNCTIONS OF THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN MALE PIGS*

for the period ending:
13th April,2017

Permit No : NACOSTI/P/16/44726/9603
Date Of Issue : 15th April,2016
Fee Recieved :Ksh 1000

Applicant's Signature

Director General
National Commission for Science, Technology & Innovation