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Hepatocyte Alterations and Impact on Reproduction among New Zealand White Female Rabbits Fed Diets with Varying Levels of Aflatoxin

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Abstract

Aflatoxin are poisonous compounds produced by fungi found in cereal grains and forage harvested or stored under humid-warm environmental conditions. Consumption of doses amounting to 20 -120 ppb of body weight per day can cause poisoning through an immediate damage on liver hepatocytes. Harmful effects of aflatoxin include their interference with both animal product production and reproductive functions. The current study sought to determine the histopathology of the liver by aflatoxin contained in the diet consumed by New Zealand white rabbits. Sixteen rabbits kept in cages were fed diets containing four levels of aflatoxin in four treatments 1 (0- level of aflatoxin), treatment 2 (100 ppb aflatoxin), treatment 3 (200 ppb aflatoxin) and treatment 4 (400 ppb aflatoxin) for a period of forty five days. The rabbits aged three months were kept in cages inside a housing structure with sufficient ventilation and 12 hour lighting daily. At the end of the feeding period, one rabbit from each treatment group was taken to anatomy and pathological laboratory, where it was humanely sacrificed and the liver harvested for histological examination. The liver tissue was sectioned and mounted on slides for microscopy. The results showed effects on the liver in all the treatment diets that contained aflatoxin. Marked focal inflammation and tissue infiltration in the periportal area of the liver were observed. The liver is the organ with the highest activation of oestrogen receptors in an animal's body, but is dependent on the intake of sufficient amount of amino acids by the animal through its diet. Sufficient amount of oestrogen receptors, activate the secretion of Estrogen receptor alpha (ERα), which expresses itself in the uterus and ovary to cause the release of estrogen and synthesis of Insulin-like growth factor 1(IGF-1), the former activates the hypothalamus to release gonadotropic releasing hormone, which target the anterior pituitary gland for the release of gonadotrophs. Aflatoxin therefore, caused inflammatory reactions on hepatocytes and cell infiltration in rabbits in treatment 2, 3 and 4, but the pathological level was higher for the latter two treatments. Rabbit diets must not contain more than 100 ppb of aflatoxin to avoid its general effects on their reproduction.

Keywords: Aflatoxin, Hepatocytes, Reproduction, Rabbits

INTRODUCTION

The changes of climatic variables during the recent few years have caused changes in the behaviour of micro-organisms. The warm-humid weather has led to the growth of moulds in crops, which exude harmful chemicals that include aflatoxin. Aflatoxins are produced in cereal grains and forages before, during and after crop harvest or stored under humid-warm environmental conditions. As a result of the diversity of their toxic effects and synergetic properties, they are a health risk to consumers of contaminated feeds (Yiannikouris & Jonany,

2002). Aflatoxin has been reported to contaminate food and feedstuff, particularly grain and nuts, at pre or post-harvest environmental conditions in tropical regions, particularly at the sub-Saharan Africa and Southeast Asia (Gong et al., 2016). The most toxic and commonly abundant aflatoxin type, which contaminates foodstuff significantly is aflatoxin B1, of which the liver is its prime target organ (Zhang et al., 2010). Recent cases of death due to aflatoxins occurred in 2016 at Tanzania. Based on past outbreaks it has been estimated that consumption for over a period of 1–3 weeks, a dose of 20–120 ppb per kg body weight per day is acutely toxic and potentially lethal (Mutegi et al., 2018). Intake of food containing concentrations of 1 ppb per kg or higher has been reported to cause high aflatoxicosis.

Aflatoxicosis in both human and animals occur either in the acute or chronic form. The acute form normally occurs upon consumption of moderate to high levels of aflatoxin over a short time. This acute form can be observed when symptoms such as haemorrhage, liver damage and disturbance of nutrient digestion, absorption or metabolism occur (Mutegi et al., 2018; Barrett, 2005). High dose consumption of aflatoxin ends up in acute poisoning that is life threatening to an animal, through its effects on the liver. Acute liver failure that is observed with signs of jaundice, nausea, lethargy and probably death, have occurred in human populations from the 1960s (Mutegi et al., 2018).

Fertility is the ability of an animal to conceive and maintain pregnancy at the appropriate time in relation to ovulation and retention of conceptus to term (Santos et al., 2010). During recent years, studies on harmful effects of aflatoxin on animals have revealed direct interference on reproduction but included indirect effects through physiological systems and their processes. More recent animal studies suggested that aflatoxin have direct effects on reproductive toxicity among both male and female animals particularly their damaging effects on gametes and physiology. In the liver, AFB1 is biotransformed by microsomal cytochrome P450 to a highly reactive intermediate, AFB1-8, 9-epoxide which binds to nucleic acids to form adducts that interfere with gametogenesis (Sun et al., 2015).

The toxic effects of aflatoxin in the liver are linked to their metabolic activation into free radical AfB1 – exo-8, 9- epoxide by cytochrome P450 enzymes and associated formation of reactive oxygen species (Saad-Hussein et al., 2019; Peles et al., 2019). The objective of the current study was to investigate the histopathology of the liver in view of its reproductive function in female New Zealand white rabbits fed on diets laced with varying concentrations of aflatoxin.

MATERIALS AND METHODS

Study Site

The current study was carried out at the University of Eldoret, within the coordinates 0°31’ N, 35°16’ E and at an altitude of 2100 m above sea level. The location has an average annual temperature of 17°C and a bimodal annual rainfall of 1100 mm. The study was carried out in a housing structure with room temperature range of 18 - 22°C and a relative humidity range of between 53 - 60%. The light in the study room was provided for 12 hours daily. The house allowed minimum air movement (wind) and was restricted from human access to minimize any possible rabbit disturbance and the risk of inhalation of aflatoxin.

Experimental Rabbits

The study used rabbits which have been regarded as useful models to extrapolate human conditions in disease situations. This is because rabbit gene expressions and functions for such factors as histopathology and immunohistochemical aspects of organs are more similar to those of human than merely phenotypic expressions (Shiomi 2009).

The New Zealand white breed of female rabbits (*Oryctolagus cuniculis*) used for the study were acquired from Tatton farm of

Egerton University demonstration and research unit in Nakuru County, who kept the breed under confinement and fed on commercial concentrate feeds. The farm produced rabbits for research, teaching and supply to both farmers and other producers. The rabbits were familiar with cage rearing and consumption of concentrate mash and/or pelleted diets. Upon arrival to the experimental site, the rabbits were each assigned a number that was written on a plastic ear tag and tagged on to the ear using an ear tag applicator. The number on the tag was engraved so that it could not be removed nor erased until the end of the study.

Housing and Care for Experimental Rabbits

The experimental rabbits were kept inside house which had wide windows closed with a combination of welded and chicken wire mesh to ensure free air circulation, but protected from entry of birds and predators. The room was reinforced against rodents, though rodenticides were used to check any possible presence of rats. The rabbit house had sufficient light through translucent iron sheets at the roof and wide windows to ensure 12 hour light: dark cycle, with a room temperature of 18 – 22^{o}C. The rabbit house was thoroughly cleaned, disinfected and anti-mite dust applied to kill any possible mites. Saw dust was spread on the floor, under the rabbit cages, before disinfection. The saw dust was meant to absorb moisture and assisted to maintain both the relative humidity and temperature at low levels and at the same time avoid offensive odour that would easily create discomfort in the room. The saw dust was removed every week to ensure the least rotting which could easily cause some infection to the rabbits.

Rabbits in each treatment were kept in cages built to measure 80 x 50 x 30 cm. These rabbit cages were built using timber frames whose sizes measured 3" x 2'', with welded wire mesh on the sides and the floor. The sides and floor of the cages constructed with welded wire mesh were reinforced with chicken wire so that the rabbit's feet don't pass through, but also walk on the floor without injury.

Feeding of the Experimental Rabbits

The persons attending to the experimental rabbits wore protective clothing that included laboratory coats, gumboots and aspirators every time while working to minimize possible entry of aflatoxin into their bodies, which has been reported to enter through wounds or nostrils by inhalation. Each treatment group of rabbits were given 400 gm of feed twice daily; at 8.00 am and 3.00 pm. The feed were provided on stable broad based earthen pots, as feed troughs, to ensure they didn't topple over incase the rabbits stepped on them. Feeds were weighed using an electronic balance before it was provided in the feed trough. Feeding was carried out twice daily to ensure that the troughs were not filled to the brim for purposes of minimizing spillage and to avail feed to the rabbits every time to ensure a stable continuous nutrient supply for body metabolic functions.

A total of sixteen - three months old rabbits were randomly distributed to four treatments using completely randomized design procedure. The experiment used 4 rabbits in each treatment in a repeated measurement approach. The rabbits were allowed seven days to familiarize with the diet, the weighing, the ear tags and the new groupings in the cages.

Harvesting and Preparation of Rabbit Organs

One rabbit was picked at random from each of the four treatments for examination of the liver to determine the effects on hepatocytes following intake of aflatoxin laced diets. A total of four rabbits were taken to the laboratory where the histological examination was carried out. The rabbits were kept in air tight glass cages for 30 minutes with a piece of cotton wool inside that was socked with 37% chloroform to bring them into unconscious state to allow humane sacrificing for the harvest of the liver. Upon harvest, the liver was preserved in 10% formalin solution before sectioning it

for microscopy. Tissue preparation for histological work was carried out in the laboratory according to procedures given by Abert et al. (2015) as stated below;

i*) Fixation of cells and tissues*

To preserve the cells within tissues, the organs were treated with a fixative. Fixatives form covalent bonds with free amino acid groups in tissues, cross linking them so that they stabilize and lock cells in their original state and positions. The freshly harvested organs and tissues were immersed into a solution with 10% formalin immediately for purposes of stabilization of cells and maintenance of their structure. Formalin was buffered and osmotically balanced to act on the tissues by minimizing shrinkage, swelling and autolysis for improved permeability into all the cells. Alcohol, at the same time removed excess water from the tissues and at the process minimized the possibility of putrefaction.

ii*) Embedding and sectioning*

Tissues and organs are soft and fragile, even after fixation they need to be embedded in a supporting medium before being sectioned. The specimens were embedded using molten paraffin wax to create a solid base that supported thin sectioning. During the embedding, tissue water was replaced with alcohol, which was also later replaced by wax that held the sectioned tissue firmly in position as it solidified. The tissue samples were thereafter sectioned into thin transparent slides of 10 µm thick using a sterile microtome blade and placed on slides. The microtome was pre-set to ensure uniformity of the sectioned slide. The sectioned tissues were laid on a slide for further processing for viewing. The slides were clearly labeled and dried at 37ºC for 1 hour to gently melt the paraffin wax and leave the tissue section intact.

iii) Staining of tissue sections

Tissue sections were stained with dyes that have specific affinity for particular sub cellular components. The sectioned tissues were stained using hematoxylin and eosin, which are the most common stain combinations for histological examinations. A cover slip was placed on top of the sectioned tissue for formation of a thin transparent film between the cover slip and the slide in readiness for microscopy.

The histological appearances of cells of the liver were examined using a binoculars microscope (Olympus CX2 Model, Japan) at magnification X40. The microscope was fitted with a digital camera that was used to take micrographs of the hepatocytes observed.

RESULTS

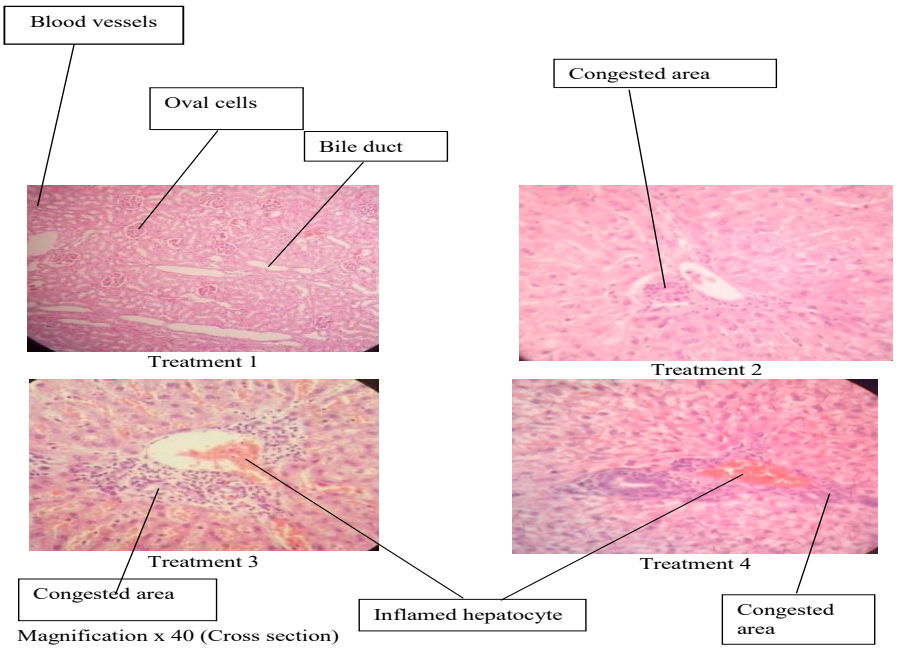

Figure 1: Liver-hepatocytes obtained from rabbits in the four treatments.

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

The photomicrograph of the liver cells in treatment 2 as indicated in figure 1, showed inflammatory reactions to aflatoxin. Focal inflammatory areas are an indication of lesions visible in the periportal area of the liver, which are indications of damage to liver hepatocytes. The dark spots are an indication of infiltration of probably lymphocytes in reactionary response to the effect of aflatoxin.

The micrographs of the liver cells in treatment 3 and 4 shown in figure 1 were indicative of inflamed area showing vascular degenerative changes of the hepatocyte cells and congestion of the periportal parts. Hepatic injury was observed as periportal inflammation and cell infiltration, bile duct proliferation, fatty acid changes and dilated central veins. Infiltration of lymphocytes and indicative inflammatory cells around the peripheral and oval cells showed an element of congestion of sinusoid. The samples from treatments 2, 3 and 4 were affected though the latter two showed higher intensity, while the rabbits in treatment 1 showed no cellular damage.

DISCUSSION

The liver is made up of 80% hepatocyte, which are the main parenchymal cells whose functions include nutrient metabolism, detoxification of poisonous compounds and the synthesis of amino acid which eventually form protein components of the body. They also capacitate body immune system against invasion by micro-organisms through secretion of immune producing proteins. Hepatocyte growth factors have been linked to the proliferation and regeneration of the endometrium and therefore are related to the involution of the uterus following parturition (Sugawara et al., 1997) and therefore

important for recovery and the initiation of the next animal cyclicity.

In the current study, aflatoxin affected the liver at all levels of inclusion in the diet, where it caused periportal inflammation, cell infiltration and general hepatic injury. Such level of pathological damage is risky for the general health and particularly reproductive function in animals. The liver is the organ with the highest activation of oestrogen receptors in an animal's body, though, it dependents on the intake of sufficient amount of amino acids by the animal through its diet (Torre et al., 2011). Sufficient amount of oestrogen receptors, activate the secretion of oestrogen receptor alpha (ERα), which expresses itself in the uterus and ovary to cause the release of estrogen and synthesis of Insulin-like growth factor 1(IGF-1), the former activates the hypothalamus to release GnRH, which targets the anterior pituitary gland for the release of gonadotrophs (Tang et al.,2019). The IGF-1 on the other hand, activates the synthesis of growth hormone whose function is the development of the uterine epithelium in preparation for the implantation of the conceptus during pregnancy (Torre et al., 2011, Tang et al., 2019). Insufficient amount of amino acid intake by animals, results in the production of oestrogen receptor Beta (ER_{β}) which downgrades the secretion of oestrogen and therefore oestrus cycle and any reproductive process in animals is terminated (Tang et al., 2019).

Toxicity of aflatoxin in mammals has been reported to cause damage to various organs of which the liver is the prime target (Azab et al., 2008). It has been recognized to cause serious health problems among animals, where following the early discovery of aflatoxin, FAO (2010) reported that it induces hepatic carcinoma at levels as low as 15 ppb. The cumulative effect of aflatoxin arising from intake in diets contribute to gradual deterioration of liver hepatocytes and in the long run may affect the overall health and fertility of the consumer (Jones et al., 1994). Aflatoxin has been reported to affect reproduction through interruption with synthesis, metabolism and interference with the steroid receptors, adverse effect on oocyte development and maturation (Santos et al., 2013). The effect on hepatocytes in the current study, may have been deleterious for reproduction particularly when it affected the receptors and oocytes and may be worst if the animal consumed it over a longer period causing chronic effect from consistent deposition on the target organ. Inflammatory reactions in the liver or ovary could affect oestrogen secretion and /or oestrogen receptors, which may result into infertility among animals. Reports indicate that any abnormal function of oestrogen synthesis is correlated to endocrine diseases and endometritis, which has been linked to fertility reductions among women and animals with liver diseases (Tang et al., 2019; Pu et al., 2020).

Aflatoxin has been reported to induce oxidative damage through the generation of free radicals which react with cellular components in the body to cause histological changes in liver functions (Peles et al., 2019). Toxins that affect protein components of this organs affect enzyme production, particularly CP450 family which is produced from the liver and kidney cells. In their findings, Yassein & Zghair (2012) reported low levels of this enzyme on damaged liver following consumption of aflatoxin. The enzyme is important for the detoxification of toxic complex compounds and its absence could result into degeneration of hepatocyte cells, which may progress to chronic form and cancerous state.

The findings of degeneration of liver cells, periportal inflammation, cell infiltration and bile duct proliferation observed in the current study concurred with those of Yassein & Zghair (2012) who observed lesions characterized by vascular degeneration in the cytoplasm of the hepatocytes with some cells showing apoptosis. This researcher also observed an aggregation of macrophages but scattered liver parenchymal cells around the central vein and fatty changes in the

cytoplasm of the hepatocytes, an indication of the dissolution effects of aflatoxin on fats, thus affecting fat consistency.

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

The liver is a non-endocrine organ which synthesizes certain hormones and hormone precursors and at the same time play a vital role in the regulation of hormone balance in an animal's body. Cellular hepatic damage, could inhibit enzyme actions or lipid and fatty acid synthesis that is necessary for gonadal or gonadotropin hormone biosynthesis (El Mahady et al., 2015). Among other important hormones, it synthesizes insulin like growth factor, which is important in the regulation of cell growth and development through stimulation of growth hormone. On the other hand, about 80% of cholesterol, a molecule necessary for the formation of steroid hormones, is produced in the endoplasmic reticulum of the hepatic cells of the liver (Santos et al., 2013).

CONCLUSION AND RECOMMEDATION

In conclusion, aflatoxin inclusion in rabbit diets at all levels in the current study had pathological effects on the liver hepatocytes, which could have been deleterious to the reproductive function of the rabbit. The study recommends that rabbit diets must not contain more than 100 ppb of aflatoxin to avoid its general effects on their reproduction.

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