AFLATOXIN M1 INDUCING GENOTOXICITY AND PATHOLOGICAL LESIONS IN ORGAN MEATS, LIVER OF CATTLE SAMPLED FROM EL-BASATIN ABATTOIR, EGYPT

Islam Elgohary[1]; Reham M. Abd-Elsalam[2]; Nasser Gamal Fadal[1]; Mahmoud, A.M. [2]*

Address(es):
[1]Department of Pathology, Animal Health Research Institute, Dokki, ARC, Giza, 12619, Egypt.
[2]Department of Pathology, Faculty of veterinary Medicine, Cairo University, Giza, 12211, Egypt.

*Corresponding author: mahmoudaly@cu.edu.eg

ABSTRACT

In this study, two hundred and thirteen liver samples were randomly collected from different ages and both sexes of cattle carcasses in El-Basatin abattoir, Cairo governorate, Egypt. One hundred ninety- seven samples out of the examined specimens were free from any bacterial infections; but showed enlargement and pallor. Thirty-nine specimens representing about 20% of the bacteria- free specimens were examined to detect the Aflatoxins and its metabolites using thin layer chromatography (TLC) and LC MS/MS. All Pathological appraisals were recorded and the genotoxicity of the studied mycotoxin was detected using single cell gel electrophoresis (Comet). Our study revealed that there were prominent deleterious effects of Aflatoxin M1 on the hepatic tissue. The detection of aflatoxins' metabolites in the hepatic tissues is of great importance on the health of human consuming organ meat; and on the other hand, it may affect the farm animals' performance.

Keywords: Aflatoxins, liver, TLC, LC MS/MS, Comet, Cattle, Histopathology

INTRODUCTION

The liver plays a vital role in mammalian digestion and metabolism; therefore, any fault in the hepatic tissue will effect on the animal health status initiating severe economic losses in farm animal production. Aspergillus fungi, particularly Aspergillus flavus and Aspergillus parasiticus, create aflatoxins (AFs), which are naturally occurring mycotoxins, it grows in stored food commodities (**Grassi *et al.,* 2007; Bryden, 2012; Streit *et al*., 2012**). Aflatoxins had hepatotoxic, carcinogenic, teratogenic, mutagenic and immunosuppressive action (**Oguz *et al.,* 2003**) and also decrease reproduction of animals (**van de Walle *et al*., 2010**). In addition to, synergistic action with various pathogenic agents (**Mozafari et al. 2017; Imran et al., 2019; Saleemi et al., 2020**); aflatoxins decrease feed utilization, poor weight gain, gastrointestinal dysfunction, kidney damage, embryonic death, reduced productivity, anemia, jaundice and death (**Pier, 1992**). About 17 recorded isolated types of aflatoxins , only 4 of them; B1, B2, G1 and G2; are studied extensively from a toxicological point of view; *Aspergillus flavus* produce only aflatoxins B1 and B2, whereas *Aspergillus parasiticus* produce aflatoxins B1, B2, G1 and G2. In humans and animals, aflatoxin M1 is a metabolite of aflatoxin B1. In the milk of cattle given contaminated diets, aflatoxin M2 is a metabolite of aflatoxin B1 (**Dhanasekaran, 2011**). Swine, chickens, turkeys, and ducklings are more susceptible to aflatoxicosis comparing with sheep and cattle which are more resistant. After ingestion of aflatoxins, they were swiftly absorbed from the gastrointestinal tract then bio-transformed by cytochrome P450 (hepatic mixed-function oxidase system) producing highly reactive epoxides which bound the nucleophilic cell sites forming adducts causing impairment of protein, DNA and RNA synthesis (**Benkerroum, 2020**). Environmental stress, sex, age, and breed variations can also additionally play a position of their toxicities (**Richard, 2008; Parsons and Munkvold, 2010; Rawal *et al*., 2010**). Also, the use of improper stored forage (silage), roughages and cereals may increase the possibility of Aflatoxicosis in ruminants (**Binder *et al.,* 2007; Keller *et al.,* 2013; Storm *et al.,* 2014**)

The liver is the essential target organ of acute aflatoxicosis in all species. It has been known that acute, subclinical and chronic disease forms have been widely described as clinical aflatoxicosis forms (**Benkerroum, 2020**). Many outbreaks of acute aflatoxicosis had been reported in bovines (**Kaleibar and Helan, 2013; Hernandez-Valdivia et al., 2020**) and the most relevant clinical signs and lesions were fatty liver and coagulation disability (**Newman et al., 2007**). Moreover, exposure to high level of aflatoxin causes alteration in digestion, absorption and metabolism of nutrients (**Van de Walle *et al*., 2010**). Chronic aflatoxicosis is similar but less evident (**Hernandez-Valdivia et al., 2020**). Chronic or subclinical exposure does not cause visible signs, but leads to carcinogenesis, metabolites of aflatoxins can intercalate into DNA (**Bennett & Klich, 2003**).

In Egypt, Liver is an edible part and could be consumed in different forms of cooking. So, in this study, great attention has been considered to detect the aflatoxin metabolites in the hepatic tissue with special references to pathological and genotoxicity of such toxins on the cattle carcasses.

MATERIAL AND METHODS

Sampling

During the period extended from August 2014 to August 2015, two hundred and thirteen liver samples from different ages and both sexes of cattle carcasses were randomly collected from El-Basatin abattoirs, Cairo governorate, Egypt. After excluding samples contained bacterial and parasitic infection either by isolation and/or detection, the remaining One hundred ninety- seven samples were grossly examined and thirty-nine samples representing 20% were presented for toxicological examination. Each specimen was divided into three parts, 1st part was collected into plastic bag for detection of aflatoxins by thin layer chromatography and LC MS/MS, 2nd part collected in phosphate buffer saline for single cell gel electrophoresis (comet) and 3rd part was fixed in 10% neutral buffer formalin for histopathological examination.

Thin Layer Chromatography (TLC)

The thin layer chromatography was performed according to (**Richard *et al.,* 1993**). Representative positive samples for aflatoxins by TLC were examined using liquid chromatography- mass spectrometry (LC MS/MS) to quantify the mycotoxin concentration in the hepatic tissue.

LC MS/MS

The representative positive samples by TLC were examined using LC MS/MS according to (**José Diana et al., 2009**) where the obtained solution was centrifuged for 10 min at 14000 g in a Ultrafree® MC centrifuge then subjected to LC-MS/MS analysis.

Single Cell Gel Electrophoresis (COMET)

Comet assay was performed according to **(Singh *et al.,* 1988; Rojas and Lopez, 1999)**. The grading of destruction DNA damage was divided into 5 grades, depending on tail length as a proportion of total length. Grade 0 with no damage, tail length <5%. Grade I with slightly damaged, tail length 5 to 20%. Grade II with moderately damaged, tail length 20 to 40%. Grade III with heavily damaged, tail length 40 to 95%. Grade IV: totally damaged, tail length >95%.

Histopathological examination

Specimens from livers were immediately fixed at 10% neutral buffer formalin,trimmed, washed in water, dehydrated in ascending concentrations of ethanol, cleared in xylene and embedded in paraffin. Thin sections (4-6 μ) were prepared and stained with hematoxylin and eosin. Masson's trichrome stain was used for staining of fibrous connective tissue **(Bancroft, 2008).**

Statistical analysis

The data were statistically analyzed using ANOVA **(Kotz and Johnson, 1982)**

RESULTS AND DISCUSSION

RESULTS

Thin layer chromatography (TLC)

31samples out of 39 liver samples were positive for detection of aflatoxins in a percentage of 79.48%. The positive results were detected by the formation of bands by using TLC. Figure 1 demonstrated the positive results.
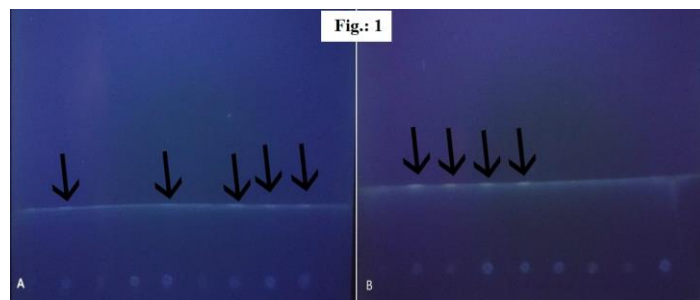

Figure 1 Thin layer chromatography showing positive nine liver samples, A, Bands of positive five samples for aflatoxins. B, Bands of Positive four samples.

LC MS/MS

Nine samples constituting 30% (representative samples) out of 31 positive samples (in TLC) showed different concentrations of aflatoxin's metabolites. The results of such concentrations are presented in Table 1.

Table 1 Concentrations of mycotoxins metabolites using LC MS/MS:

NO. of sample	Type of toxins	Conc. ppb (ng/g)
1	Aflatoxin M1	21.23
2	Aflatoxin M1	11.97
3	Aflatoxin M1	93.25
4	Aflatoxin M1	81.73
5	Aflatoxin M1	183.01
6	Aflatoxin M1	83.06
7	Aflatoxin M1	78.2
8	Aflatoxin M1	105.79
9	Aflatoxin M1	86.81

Comet assay

The interpretation of Comet assay depended on tail formation on gel electrophoresis was summarized in Table 2.

Table 2 Comet length, head diameter, DNA % in head, tail length, DNA% in tail

Examined parameter	Sample 1 *AFS 21.23 ng/gm	Sample 2 AFS 11.97 ng/gm	Sample 3 AFS 93.25 ng/gm	Sample 4 AFS 81.73 ng/gm	Sample 5 AFS 183.01 ng/gm	Sample 6 AFS 83.06 ng/gm	Sample 7 AFS 78.2 ng/gm	Sample 8 AFS 105.79 ng/gm	Sample 9 AFS 86.81 ng/gm
Comet length	18.84± 0.34 A	19.1± 0.35 B	17.3± 0.38 Cab	17.82± 0.32 Dab	18.62± 0.37 Ec	19± 0.38 Fcd	21.34± 0.3 Gabcdef	14.96± 0.38 Habcdefg	28.7± 0.24 Abcdefgh
Head diameter	15.62± 0.38 A	17.36± 0.37 Ba	15.88± 0.49 Cb	16.58± 0.35 D	17.52± 0.36 Eac	14.88± 0.47 Fbde	17.14± 0.36 Gacf	14.52± 0.4 Habcdeg	23.56±0.26 abcdefgh
DNA % in head	95.49± 0.4 A	91.38± 0.27 Ba	97.18± 0.54 Cab	95.59± 0.38 Dbc	95.46± 0.47 Ebc	91.59± 0.3 Facde	94.97± 0.36 Gbcf	95.15± 0.41 Hbcf	89.53±0.31 Abcdefgh
Tail length	3.33± 0.62 A	2.24± 0.7 B	2.24± 0.5 C	1.88± 0.58 D	2.1± 0.6 E	4.1± 0.49 Fbcde	4.2± 0.42 Gbcde	0.94± 0.89 Hafg	5.16± 0.39 Abcdeh
DNA % in tail	4.51± 0.4 A	8.62± 0.27 Ba	2.82± 0.54 Cab	4.41± 0.38 Dbc	4.54± 0.47 Ebcfi	8.41± 0.3 Fabcde	5.03± 0.36 Gbcf	4.85± 0.41 Hbcf	10.47±0.31 Abcdefgh

Legend: *AFS: Aflatoxin's metabolite concentration. Each value represents mean ± SE; n= 18. Small letters a,b,c,d,e,f,g,h,i in the same rows represents a significant change to capital letters A,B,C,D,E,F,G,H,I respectively by LSD using ANOVA at P≤ 0.05

The results were categorized according to the tail length as shown in Figure 2. The findings showed that, DNA of all samples were affected by aflatoxin but some of them were with wide variety of destruction as low level of damages (Figures 2A, 2B, 2C, 2E) while others showed high damage (Figures 2D and 2F).
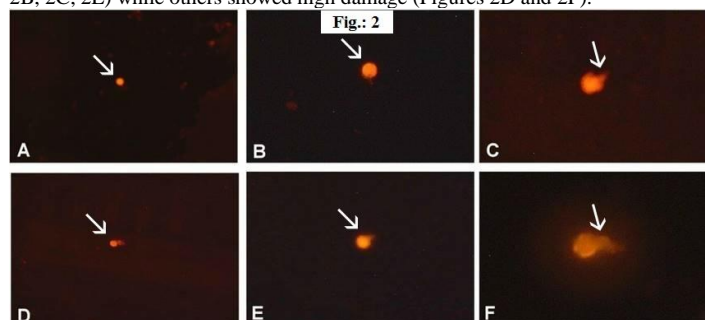

Figure 2 DNA damage detected by comet assay. A. Picture showing category 1, cell with low level of damage, head diameter 16.2 μm and tail length 2.52 μm; B.

Picture showing category 1, cell with low level of damage, head diameter 14.76 μm and tail length 0.72 μm; C. showing category 1, cell with low level of damage, head diameter 25.56 μm and tail length 3.6 μm; D. Picture showing category3, cell with high damage level, head diameter 16.2 μm and tail length 15.48 μm; E. Picture showing category1, cell with low level of damage, head diameter 31.68 μm and tail length 6.48μm; F. Picture showing category 3, cell with high damage level, head diameter 27.72 μm and tail length 18.72 0μm.

Regarding tail length in liver cells of cattle is significantly the highest in liver cells have concentration of of 86.81 ng/ gm Aflatoxin M1, then in liver cells have concentration of 78.2 ng/ gm Aflatoxin M1, then in liver cells have concentration of 83.06 ng/ gm Aflatoxin M1, then in liver cells have concentration of 21.23 ng/ gm Aflatoxin M1, then in liver cells have concentration of 93.25 ng/ gm Aflatoxin M1 equal to concentration of 11.79 ng/ gm Aflatoxin M1, then in liver cells have concentration of 183.01 ng/ gm Aflatoxin M1, then in liver cells have concentration of 81.78 ng/ gm Aflatoxin M1 and then in liver cells have concentration of 105.79 ng/ gm Aflatoxin M1.

Pathological findings

Gross examination

The examined liver samples of some cases were apparently normal but others were pale and enlarged with sub capsular hemorrhage in some areas. The hepatic tissues of such cases were friable and appeared with focal areas of necrosis. Thickening of hepatic capsule was not common.

Histopathological findings

The positive aflatoxins samples showed that, some hepatocytes were vacuolated (Figure 3A) while others showed severe lesions characterized by necrosis. Necrobiotic changes in the hepatocytes were common finding in the area of necrosis where marked pyknotic and karyolitic nuclei were noticed (Figure 3B). The hepatic capsule in some examined cases showed marked thickening with fibrosis indicating Glisson's cirrhosis (Figure 3C). In other cases, congestion in hepatic sinusoids with activation of von Kupffer cells was prominent (Figure 3D).
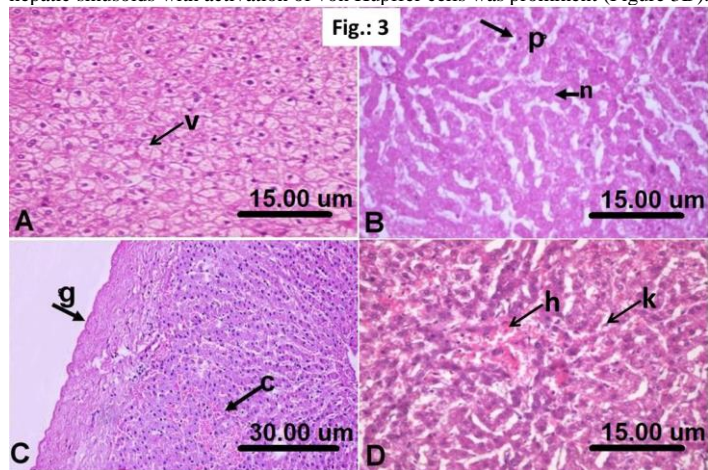

Figure 3 Histopathological section of liver (H&E stain) from positive aflatoxins samples showing, A. Hepatic tissue showing vacuolar degeneration (v) in hepatocyte with pyknotic nuclei; B. Hepatic tissue showed necrobiotic changes of hepatocyte (n) where some hepatocytes have pyknotic nuclei (P); C. Hepatic tissue showing Glisson's cirrhosis (g) and congestion in hepatic sinusoids (c); D. Hepatic tissue showing focal areas of hemorrhages (h) and activation of von Kupffer cells (k).
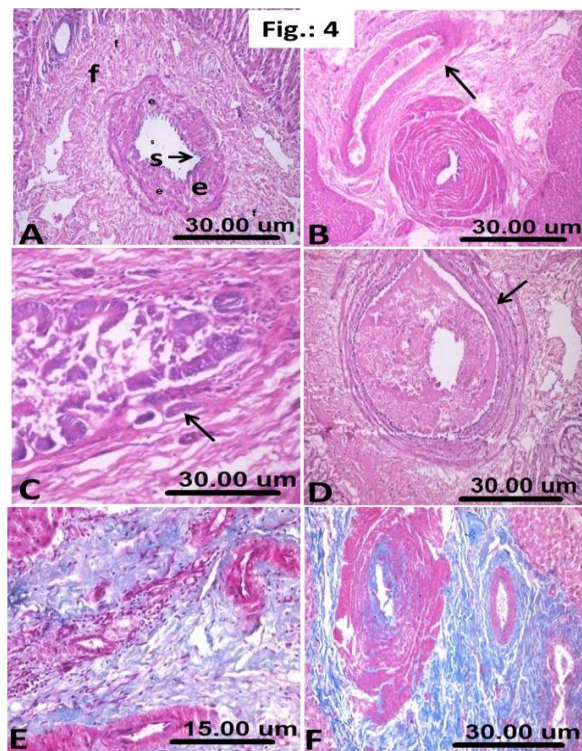

Figure 4 Histopathological examination of positive aflatoxins samples (A-D, H&E stain, E-F, Masson's trichrome stain). A. Hepatic tissue showing portal fibrosis characterized by proliferation of fibrous connective tissue (f). Notice: Endophelibitis in hepatic vein (e) with swelling of endothelium of these veins (s); B. Hepatic tissues showed sever portal fibrosis (arrow), endphlebitis in hepatic

veins, congestion and dispersion of the structural elements in the wall of portal blood vessels (arrow); C. Hepatic tissue showing hyperplasia in epithelium lining bile duct with formation of newly formed bile ductule (arrow) and characteristic portal fibrosis; D. Hepatic tissue showing portal fibrosis and thrombus in portal blood vessel (arrow). The thrombus attached to the endothelium; E. Hepatic tissue showing portal fibrosis, the proliferated fibrous connective tissue stained blue while the wall of portal blood vessel appeared vacuolated; F. Hepatic tissue showing fibrous connective tissue (blue color) and presence of endophlebitis in hepatic veins (red color).

Portal fibrosis with endophlebitis in hepatic vein was characteristic. Swellings in endothelium of these veins in such cases were observed (Figure 4A). The portal blood vessels sometimes showed dispersion of the structural elements of its wall (Figure 4B).). Epithelial hyperplasia of some bile ductules with formation of newly formed bile ductules was also observed (Figure 4C). Some of th hepatic veins showed thrombus formation (Figure 4D). Masson's trichrome staining of hepatic tissue was performed to assess the collagen fibre distribution in the liver tissue (Figures 4E and 4F).

DISCUSSION`

In our study, 31samples out of 39 were positive for aflatoxin (79.48%) by using TLC. The concentration of aflatoxin M1 in representative samples using MS/MS indicates different concentration values. The gross examination of these samples showed liver enlargement, pale color and in some cases presence of subcapsular hemorrhages. Such findings were agreed with **(Pereyra et al., 2008)** who showed that the livers of animals affected by aflatoxicosis revealed size enlargement, pale coloration, rounded borders, and expanded friability.

The histopathological examination revealed that hepatocytes showed multiple vacuoles while other hepatocytes showed necrobiotic changes as pyknosis and karyolysis which were in agreement with previous studies of **(Colakoğlu and Donmez, 2012)** who showed that Vacuolar degeneration and pyknotic nuclei in the hepatocytes in some lobules were common in aflatoxicosis. The same results were achieved by **(Devendran et al., 2011)** who showed the effect of aflatoxin on the hepatocytes was appeared as severe hydrophilic and vacuolar degeneration in the most of hepatocytes with disseminated necrotic cells.

In the present study, Portal fibrosis and endophelibitis were the common findings which characterized by swelling of endothelial cells and marked thickening in hepatic vein wall that lead to obstruction of hepatic vein in some cases. In addition to that, the bile ducts were hyperplasic with newly formed bile ductules. The same results were recorded by **(Colvin et al., 1984)** who discussed the Aflatoxicosis in feeder cattle and showed that presence of portal fibrosis with biliary proliferation and venous obstruction as the disease progresses. This results were also in agreement with previous studies of **(Rajendran et al., 1992)** who made Clinicopathology of aflatoxin toxicity in cattle and showed proliferation of bile duct epithelium, obliterating endophlebitis of centrilobular and hepatic veins (characteristic to bovines) and diffuse fibrosis leading to variation in morphology of hepatocytes.

the positive aflatoxin samples appear as bands by using ultraviolet lamb which in agreement with **(Grosso et al., 2004)** who proved that TLC is used to identify and quantitate aflatoxins at very low levels and confirmed by **(Trucksess, 2000)** who determined the aflatoxins in chinchilla feed samples.

The concentration and type of aflatoxins are determined in liver specimens by LCMSMS where high concentration of aflatoxin M1 (AFM1) in these liver sample (Table 1)**.** The mycotoxin metabolite concentration exceeding the maximum level of AFM proposed by the WHO (0.500 µg AFM1/kg), the European Regulation (EC) declares a limit of 0.050 µg AFM1/kg milk (**Commission Regulation (EC) 2006)**.

Comet results in our study showed destruction on DNA of hepatocytes in the form of head and tail due to Aflatoxins can induce mutagenesis by alkylation of nuclear DNA, leading to carcinogenesis and teratogenesis which is go parallel with the findings of **(Hussain et al., 2007)** who showed that the carcinogenic and teratogenic effect occurred due to The reaction with DNA occurs with guanine in the codon 249 of tumor suppressor gene p53 where the G to T trans version occurs. This result was also in agreement with **(Wong and Hsieh, 1976)** who proved that the limited ability to metabolize AFM1 into the DNA-reactive epoxide may thus account for its ability to cause DNA damage and pre-neoplastic lesions as compared to AFB1.

From statistical point of view, our study revealed that, some of the examined samples showed marked increase of the DNA fragmentation with the increased mycotoxin concentration as in sample No. 5 in where the mycotoxin concentration was 183.01 ng/gm the tail length in comet assay was 2.1± 0.6, in sample No. 8 where the concentration was 105.78 ng/gm and tail length was 0.94± 0.89 on the other hand in other specimens although the concentration of mycotoxins were lower as in sample No. 2,1,7,4 and 9 where the mycotoxin concentration was (11.79, 21.23,78.2,81.73and 86.81ng/gm) respectively , the tail length in comet assay was (2.24± 0.7, 3.33± 0.62, 4.2 ± 0.42, 1.88 ± 0.58 and 5.16± 0.39). In this concern the role of sex, age, environmental stress and breed differences may play role in its toxicities which confirmed by **(Richard, 2008; Parsons and Munkvold, 2010; Rawal *et al*., 2010)**.

Also, the animal could be ingested concentrates of high mycotoxin content just before slaughtering where the genotoxic effect of such toxins not allowed to appear in examined cases while the concentration of the mycotoxin's metabolite appeared in a higher value. The comparative carcinogenicity of AFM1 and AFB1 was detected in both vivo in rat models and in vitro, in murine and human liver microsomes **(Neal et al., 1998)**.

The decreased extent of DNA damage and pre-neoplastic lesions that were observed in a case of AFM1, may be due to its limited ability to metabolize it DNA-reactive epoxide when compared with AFB1. AFM1 is mutagenic in vitro in Salmonella typhimurium strains **(Wong and Hsieh, 1976)** and showed the same in vivo genotoxic potential as AFB1 in Drosophila melanogaster **(Shibahara et al., 1995)** indicating a possible in vivo genotoxicity and mutagenicity in mammalians.

After consumption of mycotoxins, the biotransformation of aflatoxins is to make it hydrophilic to be excreted through urine and bile. These biotransformation of AFB1 occur either by cytochrome reductase enzyme to form aflatoxicol or by liver microsomal enzyme in form of epoxidation to form AFB_1-epoxide which responsible for carcinogenesis, hydration to form of AFB_{2a} which responsible for acute toxicity and liver necrosis, hydroxylation to form AFM_1 + AFQ_1 which excreted in bile and urine and o-demethylation to form AFP_1 which excreted in bile and urine **(Dhanasekaran et al., 2011)**. AFM1 is normally detected in milk within 12 h of administration of AFB1-contaminated feed **(Diaz et al., 2004)**.

As a result of continuous daily exposure to stable levels of AFB1, the concentration of AFM1 in milk increases linearly for several days before finally reaching a steady state, equilibrium between intake and excretion occur **(Battacone et al., 2003)**. Consequently, other multi-species studies have confirmed the hepatotoxic and carcinogenic effects of AFM1 compared to AFB1 **(WHO, 1993)**.

In terms of human health hazards, aflatoxins affect human health due to the fact that most mycotoxins are heat tolerant in the normal food processing temperature range (80–121 ∘ C), so the result is a slight decrease in the total level of the toxin or its absence. under normal cooking conditions such as boiling and frying, or even after pasteurization **(Smith et al., 1994)**. Although aflatoxins are highly stable to dry heat and have high decomposition temperatures ranging from 237 to 306 degrees Celsius, many attempts have been made to inactivate aflatoxins in various foods. It has been reported that temperatures above 150°C are required to partially destroy the toxin **(Samarajeewa et al., 1990)**. Initially, AFM1 was categorized as group 2B human carcinogen by IARC **(WHO, 1993)**. Other improved studies reclassified AFM1 as a group 1 human carcinogen **(WHO, 2002)** while it was demonstrated that AFB1 must be converted into its reactive epoxide to bind protein and exert acute toxic effects **(Neal et al., 1981)**, this process does not seem crucial to the cytotoxicity of AFM1.

In human cell lines (MCL5), whether or not they express the CYP enzyme, AFM1 showed the potential for direct toxicity in the absence of metabolic activation, in contrast to AFB1 **(Neal et al., 1998)**. Recently, direct cytotoxicity of AFM1 has been identified in cultured human enterocytes (Caco-2) **(Zhang et al., 2015)**.In Kenya, young children are weaned at an early age **(Bwibo and Neumann, 2003)**; consumption of AFM1-contaminated milk makes them more susceptible to other diseases due to reduced immune development.

CONCLUSION

From this study, we could conclude that the detection of the aflatoxin and its metabolites in the edible meat, organ (liver) of cattle is a serious problem and it caused cellular and DNA damage in hepatocytes. As the aflatoxin is metabolized in the liver of livestock and the metabolites should exist in the hepatic tissue for instance prior excretion through milk. So, the liver may consider as an important source of such toxin metabolites for human consuming the organ meats. Also, it is well known that aflatoxins and their metabolites are heat resistant and so, it could be threatened the human health.

REFERENCES

Bancroft, J.D., 2008. Theory and Practice of Histological Techniques. Elsevier Heal Sci. UK.;6th Edn.

Battacone, G., Nudda, A., Cannas, A., Borlino, A. C., Bomboi, G., and Pulina, G., 2003. "Excretion of Aflatoxin M1 in Milk of Dairy Ewes Treated with Different Doses of Aflatoxin B1," *Journal of Dairy Science*, Vol. 86, No. 8, pp. 2667-2675. https://doi.org/10.3168/jds.S0022-0302(03)73862-4

Benkerroum N, 2020. Chronic and Acute Toxicities of Aflatoxins: Mechanisms of Action. Int J Environ Res Public Health 17:423:1- 28. https://doi.org/10.3390/ijerph17020423

Bennett, J.W., and Klich, M., 2003. Mycotoxins. *Clin Microbiol Rev*.;16(3):497–516.

Binder E.M., Tan L.M., Chin L.J., Handl J., Richard J., 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim. Feed Sci. Technol., 137:265–282. https://doi.org/10.1016/j.anifeedsci.2007.06.005

Bryden, W.L., 2012. Mycotoxin contamination of the feed supply chain: Implications foranimal productivity and feed security. Animal Feed Science and Technology 173: 134– 158. https://doi.org/10.1016/j.anifeedsci.2011.12.014

Bwibo, N.O., and Neumann, C.G., 2003. The need for animal source foods by kenyan children. *J. Nutr*., 133, 3936S–3940S. https://doi.org/10.1093/jn/133.11.3936s

Colvin, B. M., Harrison, L. R., Gosser, H. S., and Hall, R. F., 1984. Aflatoxicosis in feeder cattle. *J. Am. Vet. Med. Assoc*., 184: 956- 958.

Colakoglu, F.,,and Donmez, H.H., 2012. Effects of Aflatoxin on Liver and Protective Effectiveness of EsterifiedGlucomannan inMerino Rams Fatma. *The ScientificWorld Journal*, PP.1-5. https://doi.org/10.1100/2012/462925

Commission Recommendation № 2006/576/EC (August 2006) of 17 on the Presence of Deoxyvalenol, Zearale- None, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding," *Official Journal of the European Union*, Series L, No. 229, pp. 7-9.

Devendran, G., and Balasubramanian, U., 2011. Biochemical and histopathological analysis of aflatoxin induced toxicity in liver and kidney of rat. *Asian Journal of Plant Science and Research*, 1 (4):61-69.

Dhanasekaran, D., 2011. Aflatoxins and Aflatoxicosis in Human and Animals. "Aflatoxins - *Biochem Mol Biol*. :221-255. https://doi.org/10.5772/22717

Diaz, D.E., Hagler, W.M., Blackwelder, J.R., Eve, J.T., Hopkins, J.A., Anderson, B.A., Jones, K.L., F.T.; Whitlow, L.W. 2004. Aflatoxin binders ii: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in *feed. Mycopathologia*, 157, 233–241. https://doi.org/10.1023/B:MYCO.0000020587.93872.59

Grassi, T.F., Pires, P.W., Barbisan, L.F., Pai-Saliva, M.D., Said, R.A., and et al., 2007. Liver lesions produced by aflatoxins in Rana catesbeiana (bullfrog). *Ecotoxicol Env Saf*. ;68:71-78. https://doi.org/10.1016/j.ecoenv.2006.07.007

Grosso, F., Fremy, J. M., Bevis, S., and Dragacci, S., 2004. Joint IDF-IUPAC-IAEA (FAO) inter-laboratory validation for determining aflatoxin M1 in milk by using immunoaffinity clean-up before thin-layer chromatography. *Food Addit. Contam*., 21(4): 348-357. https://doi.org/10.1080/02652030410001662048

Hernandez-Valdivia, E., Valdivia-Flores, A.G., Cruz-Vazquez, C., Martinez-Saldaña, M.C., Quezada-Tristan, T., Rangel-Muñoz, E.J., Ortiz-Martinez, R., Medina-Esparza, L.E. and Jaramillo-Juarez, F., 2020. Diagnosis of Subclinical Aflatoxicosis by Biochemical Changes in Dairy Cows under Field Conditions. Pak Vet J. http://dx.doi.org/10.29261/pakvetj/2020.075

Hussain, S. P., Schwank, J., Staib, F., Wang, X.W., and Harris, C.C., 2007. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. *Oncogene*. ;26(15):2166-2176. https://doi.org/10.1038/sj.onc.1210279

Imran M, Cao S, Wan SF, et al., 2020. Mycotoxins - a global one health concern: A review. Agrobiological Records 2: 1-16. https://doi.org/10.47278/journal.abr/2020.008

José Diana Di Mavungu, UGent, Sofie Monbaliu UGent, ML Scippo, G. Maghuin-Rogister, Y.J. Schneider, Y. Larondelle, A. Callebaut, J. Robbens, Carlos Van Peteghem UGent and Sarah De Saeger UGent, 2009. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food additives and contaminants part a-chemistry analysis control exposure & risk assessment*. 26(6). pp.885-89. https://doi.org/10.1080/02652030902774649

Kaleibar MT and Helan JA, 2013. A field outbreak of aflatoxicosis with high fatality rate in feedlot calves in Iran. Comp Clin Pathol 22:1155-63. http://dx.doi.org/10.1007%2Fs00580-012-1543-1

Keller L.A.M., González Pereyra M.L., Keller K.M., Alonso V.A., Oliveira A.A., Almeida T.X., Barbosa T.S., Nunes L.M.T., Cavaglieri L.R., Rosa C.A.R., 2013. Fungal and mycotoxins contamination in corn silage: Monitoring risk before and after fermentation. *J. Stored Prod. Res*., 52:42–47. http://dx.doi.org/10.1016%2Fj.jspr.2012.09.001

Kotz and Johnson, N.L., 1982. encyclopedia of stastical science. vol.1., John wiley and Sons, Newyork,PP.61-69.

Mozafari, S., Mohsenzadeh, M. and Mehrzad, J., 2017. Seasonally feed-related aflatoxins B1 and M1 spread in semiarid industrial dairy herd and its deteriorating impacts on food and immunity. Journal of Food Quality, 2017. https://doi.org/10.1155/2017/4067989

Neal, G.E., Judah, D.J., Stirpe, F., and Patterson, D.S., 1981. The formation of 2,3-dihydroxy-2,3-dihydroaflatoxin B1 by the metabolism of aflatoxin B1 by liver microsomes isolated from certain avian and mammalian species and the possible role of this metabolite in the acute toxicity of aflatoxin B1. *Toxicol. Appl. Pharmacol*., 58, 431–437. https://doi.org/10.1016/0041-008X(81)90095-8

Neal, G.E., Eaton, D.L., Judah, D.J., and Verma, A., 1998. Metabolism and toxicity of aflatoxins M1 and B1 in human-derived in vitro systems. *Toxicol. Appl. Pharmacol*., 151, 152–158. https://doi.org/10.1006/taap.1998.8440

Newman, S.J., Smith, J.R., Stenske, K.A., Newman. L.P., Dunlap, J.R., and et al., 2007. Aflatoxicosis in nine dogs after exposure contaminated commercial dog food. *J Vet Diagn Invest*.;19:168-175. https://doi.org/10.1177%2F104063870701900205

Oguz, H., Hadiml,i H. H., Kurtoglu, V.,,and Erganis, O., 2003. Evaluation of humoral immunity of broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. *Rev deMedecine Vet*.;154(7):483-486.

Parsons, M.W., and Munkvold, G.P., 2010. Associations of planting date, drought stress, and insects with Fusarium ear rot and fumonisin B1 contamination in California maize. *Food Addit. Contam*. 27, 591–607. https://doi.org/10.1080/19440040903456337

Pereyra, M. L. G., Carvalho E. C. Q., Tissera J. L., Keller K. M, Magnoli C. E., Rosa C. A. R., Dalcero A. M., and Cavaglieri L. R., 2008. An outbreak of acute aflatoxicosis on a chinchilla (Chinchilla lanigera) farm in Argentina. *J Vet Diagn Invest* 20:853–856 https://doi.org/10.1177%2F104063870802000629

Pier, A.C., 1992. Major biological consequences of aflatoxicosis in animal production. *J. Anim. Sci*. 70:3964–3967. https://doi.org/10.2527/1992.70123964x

Rajendran, M. P., Sundararajan, S., Chennakesavanu, M., Samuel, C.Y., and Sundararaj, A., 1992. Clinicopathology of aflatoxin toxicity in cattle. *Indian Vet J*.;69:115-117.

Rawal, S., Kim, J.E., Coulombe R.jr., 2010. Aflatoxin B1 in poultry: toxicology, metabolism and prevention. *Res. Vet. Sci*. 89, 325–331. https://doi.org/10.1016/j.rvsc.2010.04.011

Richard, J.L., 2008. Discovery of aflatoxins and significant historical features. *Toxin Rev*.;27:171-201. https://doi.org/10.1080/15569540802462040

Richard, J.L., G.A. Bennett, P.F. Ross, and P.E. Nelson, 1993. Analysis of naturally occurring mycotoxins in feedstuffs and food. *J Anim Sci*.;71(9):2563-2574. https://doi.org/10.2527/1993.7192563x

Rojas, E. and M.C.V.M. Lopez, 1999. Single cell gel electro phoresis assay: methodology and applications. *J Chromatogr B Biomed Sci Appl*.;722:225-254. https://doi.org/10.1016/S0378-4347(98)00313-2

Saleemi MK, Ashraf MK, Gul ST, et al., 2020. Toxicopathological effects of feeding aflatoxins B1 in broilers and its amelioration with indigenous mycotoxin binder. Ecotoxicology and Environmental Safety 187. https://doi.org/10.1016/j.ecoenv.2019.109712

Samarajeewa,U,, Sen, A.C., Cohen, M.D. and Wei, C.I., 1990. Detoxification of aflatoxins in foods and feeds by physical and chemical methods. *J Food Protect* 53:489–501. https://doi.org/10.4315/0362-028X-53.6.489

Shibahara, T., Ogawa, H.I., Ryo, H., Fujikawa, K., 1995. DNA-damaging potency and genotoxicity of aflatoxin M1 in somatic cells in vivo of Drosophila melanogaster. *Mutagenesis* 10, 161–164. https://doi.org/10.1093/mutage/10.3.161

Singh, N,P,, M.T. McCoy, R.R. S..Tice, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*.;175:184−191. https://doi.org/10.1016/0014-4827(88)90265-0

Smith, E,E,, Phillips, T.D., Ellis, J.A,, Harvey, R.B., Kubena, L.F., Thompson, J. and Newton, G., 1994. Dairy goat milk and effects on milk production and components Dietary Hydrated Sodium Calcium Aluminosilicate Reduction of Aflatoxin M1 Residue in Dairy Goat Milk and Effects on Milk Production and Components. *Journal of Animal Science*, 72:677-682. https://doi.org/10.2527/1994.723677x

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., and et al., 2012. Current Situation of Mycotoxin Contamination and Co-occurrence in Animal Feed—Focus on Europe. *Toxins*, 4(10), 788-809. https://www.mdpi.com/2072-6651/4/10/788#

Storm I.M., Rasmussen R.R., Rasmussen P.H., 2014. Occurrence of pre- and post-harvest mycotoxins and other secondary metabolites in Danish maize silage. *Toxins* (Basel); 6(8):2256-69. https://doi.org/10.3390/toxins6082256

Trucksess, M.W. 2000. Natural toxins. In: AOAC official methods of analysis, ed. Horwitz W, 17th ed., p. 16. AOAC International, Gaithersburg, MD.

van de Walle, J., Sergent, T., Piront, N., Toussaint, O., Schneider, Y.J. and et al., 2010. Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. *Toxicol. Appl. Pharmacol*. 245, 291–298. https://doi.org/10.1016/j.taap.2010.03.012

World Health Organization (WHO); 1993. International Agency for Research on Cancer. Aflatoxins. In IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; IARC Press: Lyon, France, Volume 56, pp. 245–395.

World Health Organization (WHO); 2002. International Agency for Research on Cancer, Aflatoxins. In IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; IARC Press: Lyon, France,; Volume 82, pp. 171–300.

Wong, J.J., and Hsieh, D.P., 1976. Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. *Proc. Natl. Acad. Sci. USA*, 73, 2241–2244. https://doi.org/10.1073/pnas.73.7.2241

Zhang, J., Zheng, N., Liu, J., Li, F.D., Li, S.L., and Wang, J.Q., 2015. Aflatoxin B1 and aflatoxin M1 induced cytotoxicity and DNA damage in differentiated and undifferentiated Caco-2 cells. *Food Chem. Toxicol*., 83, 54–60. https://doi.org/10.1016/j.fct.2015.05.020