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

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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 affect 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 (van de Walle et al., 2010). Due to their toxicity, aflatoxins can intercalate into DNA (Bennett & Klisch, 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 liquid chromatography 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 I with no damage, tail length <5%. Grade II 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)

31 samples 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:

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

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

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

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 18.72µm. Regarding tail length in liver cells of cattle is significantly the highest in liver cells have concentration of 86.81 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 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](image)

**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).

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 alklation of nuclear DNA, leading to carcinogenesis and teratogenesis which is go parallel with the findings of (Wong and Hsieh, 1976) 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 chunchilla 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).

In our study, 31 samples 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 (Percyra 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 pyknotic and karyolitic nuclei which were in agreement with previous studies of (Colakoglu 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 (Devendra et al., 2011) who showed the effect of aflatoxin on the hepatocytes was as observed was severe hydrophilic and vacuolar degeneration in the most of hepatocytes with disseminated necrotic cells.

In the present study, Portal fibrosis and endophlebitis 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 hyperplastic 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 and hiliar proliferation and venous obstruction as the disease progresses. This results were also in agreement with previous studies of (Rajendra et al., 1992) who made Clinocopathology of aflatoxin toxicity in cattle and showed proliferation of bile duct epithelium, obliteration 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 chunchilla feed samples.

DISCUSSION

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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 alklation 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 transversion occurs. This result was also in agreement with (Wong and Hsieh, 1976) who proved that the limited ability to metabolize AFM1 into the DNA and 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, 28.81,73 and 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 context, the role of sex, age, environmental stress and breed 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 rat in vivo 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 Hoieh, 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 mammals.

After consumption of mycotoxins, the biotransformation of aflatoxins is to make it drop out from the biota after excretion 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 AFM1-epoxide which responsible for carcinogenicity, hydration to form of AFB3 which responsible for acute toxicity and liver necrosis, hydroxylation to form AFM2; AFQ, 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 destroy the toxin (Samarajeeva 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 AFB1.

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 were 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.

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