Global Veterinaria 15 (1): 113-120, 2015
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2015.15.01.95302

# Some Clinicopathological, Pathological and Immunohistochemical Studies on Tissue Samples Collected From Cattle, Sheep and Goat Fed on Spoiled Silage Containing Aflatoxin B1

*[1]A.S. Galbat, [2]A.A. Madboli, [3]A. El-shemy and [2]A.H. Soror*

[1]Department of Animal Medicine, Assiut University,
Faculty of Veterinary Medicine, New Vally branch, New Vally Governorate, Egypt
[2]Department of Animal Reproduction and Artificial Insemination,
Veterinary Research Division, National Research Centre, Giza, Egypt
[3]Department of Parasitology, Veterinary Research Division, National Research Centre, Giza, Egypt

**Abstract:** The aim of this study was confirming the gaining access of aflatoxin B1 (AFB1) into testis and uterus of animals fed on spoiled silage containing aflatoxins. Seven silage samples monthly collected for 3 months from silos in Menofia governorate Egypt then pooled into three main samples. HPLC was quantified AFB1 in examined samples as 0.78, 0.8 and 54 µg/kg respectively. A history of ten cows, four bulls, three ewes and three goats were fed on the same spoiled silage containing aflatoxins for three months were recorded till slaughtered at abattoir. Tissue and serum samples were collected for clinicopathological and histopathological changes. Liver and kidney function was significantly increased; however glucose level was significantly decreased in examined animals. Immunohistochemical (IHC) examination detect and localize the AFB1 antigen in spermatocytes, Sertoli cells, epididymis in three bulls; Also detected in endometrium of seven cows and one goat. Liver and kidney showed high antigen intensity in nine cows. Degeneration and necrosis of spermatocytes and epididymis were occurred in bulls. Nine cows showed lymphocytic ulcerative endometritis. Examined ewes and goats showed mild endometritis. Hepatocytic hyperchromatosis was noticed in seven cows and periportal lymphocytosis was seen in the liver. Conclusion; AFB1 existence in bad stored silage leading to several adverse effects and can gain access to the genitalia of cows and bulls; in turn affect in the reproductive performance.

**Key words:** Aflatoxins · Clinical Pathology · Immunohistochemistry · Pathology

## INTRODUCTION

Fungal growth on silage based up on moisture, temperature and oxygen availability [1]. Aflatoxins (AFs) are a group of approximately 20 related fungal metabolites produced by Aspergillus fungi, especially *A. flavus* and *A. parasiticus* [2]. The main four known aflatoxins include Aflatoxin B1 (AFB1), AFB2, AFG1 and AFG2 [3]. AFB1 is a micro molecule reach to the respiratory system by inhalation so the food industry workers were became under risk [4]. Several studies were carried out on AFB1 with concentration on its ability to cause hepatocellular carcinoma [5].

Aflatoxin causing acute hepatic injury that leads to elevation in serum enzymes [6]. Hepatotoxicity of AFB1 leads to congestion, vacuolar degeneration, necrosis and inflammatory cell infiltration [7]. AFB1 caused infertility, abortion and hormonal disturbances [8]. Pregnant and growing animals are less susceptible than young animals, mature animals more resistant to aflatoxin [9]. AFB1 caused reproductive anomalies in mice, rats, pigs, sheep and cattle [10]. AFB1 in mice exhibited histological changes in testis as inhibition of spermatogenesis, increase sperm abnormalities; also germ cell necrosis and vacuolar degeneration in leydig cells [11].

**Corresponding Author:** A.A. Madboli, Department of Animal Reproduction and AI, National Research Center,
Tel: +201120034521, Fax: +20237601877, E-mail: abdelnasser_mazen_monzer@yahoo.com.

The main aim of this study was to carried out some clinicopathological, pathological and immunohistochemical studies on serum and tissue samples collected from Cattle, Sheep and Goat fed on spoiled silage containing aflatoxin B1.

## MATERIALS AND METHODS

**Quantification of Aflatoxins in Spoiled Silage Samples by HPLC:** Twenty one silage samples (seven samples from seven silos per month for three months) were collected from different silos in Menofia Governorate Egypt each seven samples pooled into one sample (totally the 21 silage samples pooled into 3 samples). The 3 samples were analyzed by HPLC in the Central Lab. Of Mycotoxins in National Research Center (NRC) to quantify the different aflatoxin fractions (AFG1, AFB1, AFG2 and AFB2) using AflaTest ®-P affinity column [12]. HPLC system imported from Waters Corporation Co. (USA).

**Examined Animals:** A total number of four mature bulls ten non-pregnant cows aged from three to five years, three non-pregnant ewes and three non-pregnant goats aged from two to four years, fed on spoiled silage for three months in Menofia governorate Egypt. Three control animals from the same species previously mentioned fed on formulated ration free from aflatoxins fractions were examined as control negative animal.

**Clinicopathological Examination:** Blood samples were collected from jagular vein just before slaughtering of the tested animals in abattoir. Serum glucose was measured according to Trinder [13]. AST, ALT activities carried out according to Ritman and Frankle [14]. Creatinine and Urea were done according to Oliver [15]. Reagents supplied by Biomerieux-France and randox Co.

**Statistical Analysis:** Statistical differences were calculated according to T- test with significance level at (P ? 0.05). All results were analyzed using the procedure of SAS [16].

**Histopathology:** Tissue samples as testis, epididymis, uterus, kidney and liver were fixed in 10% neutral buffer formalin (NBF) for 24 hr, routinely processed, embedded in paraffin wax and sectioned at 5 µm [17].

**Immunohistochemistry:** The tissue samples previously mentioned were fixed in 10% NBF for 24 hr. then transferred into alcohol 70% after washing by distal water. Streptavidin/biotin peroxidase detection kit imported from Scy Tek Lab. USA. Species specificity of kit is anti-mouse, anti-rabbit. Tissue retrieval was done by incubation with Proteinase K (0.1%) at 37°C/15 minute. Slides were Incubated with mouse monoclonal anti AFB1 IgG1 6A10 as primary antibody imported from Thermo Lab. India. DAB chromogen as a color indicator was applied [18].

## RESULTS

**HPLC:** HPLC analysis of the three silage samples revealed low AFB1 in the 1st and 2nd samples. The 3rd sample (after 3 month from fermentation) was dramatically increased in AFB1 as shown in table (1) and Figure (1) in comparison to standard curve.

Table 1 and figure 1 showed dramatical increase in the 3rd sample especially at the level of AFB1 (54 µg/ kg) (blue arrow) and generally at the total aflatoxins (120.32 µg/ kg) (after 3 month from fermentation).

**Clinicopathological Findings:** AST, ALT, creatinine and urea levels were significantly increased in bulls, cows, ewes and goats except samples number 1 in bulls, 6 in cows, 1 in goats and 3 in ewes. Glucose level was significantly decreased except in the same previously mentioned samples as in Tables (2).

**Histopathological and Immunohistochemical Findings. Testis and Epididymis of Bulls:** Histopathological findings in testis and epididymis of the all 4 bulls revealed severe degeneration, desquamation and necrosis in spermatocytes of seminiferous tubules. Some tubules showing multi nucleated giant cells (Fig.1). Strong positive golden brown immunoreaction against AFB1antigen was observed in Sertoli cells and most of spermatocytes in 3 bulls (Fig. 2). Epididymis showed severe degeneration, necrosis in the lining epithelium that associated with desquamation of some necrotic cells to the lumen of epididymal tubules (Fig. 3). Epididymal epithelium showed strong positivity against AFB1antigen in most of the lining epididymal tubules (Fig. 4). *Mycotic hyphae* in intertubular area were exhibited negative immunoreaction (blue color) where the anti aflatoxin B1 antibody reacts only and specifically against aflatoxin B1 antigen (Fig. 5, 6).

Table 1: HPLC analysis of silage for different fractions of aflatoxins

| Samples | Toxin fractions<br>AFG1 | ATB1 | AFG2 | AFB2 | Total aflatoxin |
|---|---|---|---|---|---|
| 1 | ND | 0.8µg/kg | 0.012µg/kg | 0.12 µg/kg | 0.842µg/kg |
| 2 | 0.3 µg/kg | 0.78 µg/kg | 3.47 µg/kg | 0.3 µg/kg | 4.625 µg/kg |
| 3 | 44 µg/kg | 54 µg/kg | 10.41 µg/kg | 11.91 µg/kg | 120.32 µg/kg |

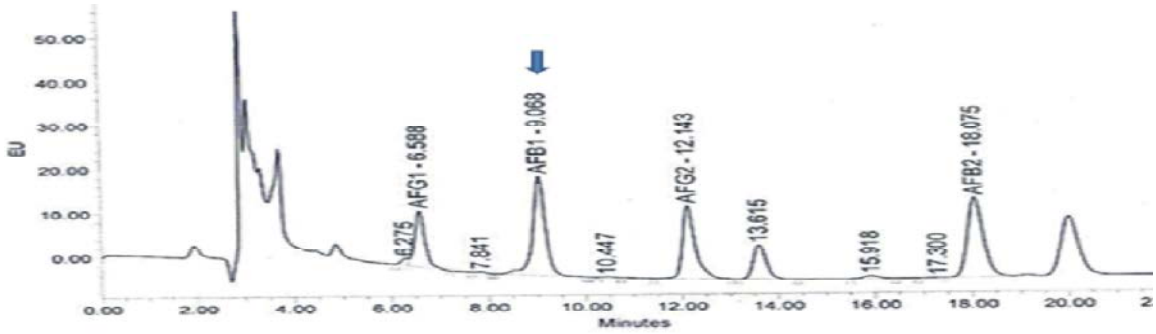


Fig. 1: HPLC analysis of silage in the 3rd sample of aflatoxins fractions

Table 2: Serum AST, ALT, Creatinine, urea and glucose levels in Bulls, Cows, Goats and Ewes fed on silage contaminated with Aflatoxin B1

| | | Liver Function | | Kidney function | | |
|---|---|---|---|---|---|---|
| Species | Cases | AST IU/L | ALT IU/L | Creatinine µg/DL | Urea µg/DL | Glucose µg/DL |
| BULL | Control negative | 62.50±6.01 | 21.40±0.40 | 0.90±0.30 | 22.60±2.54 | 73.04±9.07 |
| BULL | Case1 | 59.10±5.70 | 21.90±0.92 | 1.20±0.45 | 23.07±1.20 | 70.98±6.80 |
| BULL | Case 2 | 71.30±5.49* | 23.30±0.70* | 1.80±0.20* | 23.56±1.05* | 62.41±5.64* |
| BULL | Case 3 | 69.80±4.06* | 23.50±0.70* | 1.65±0.40* | 23.21±0.95* | 59.47±4.10* |
| BULL | Case 4 | 75.00±6.03* | 25.40±0.58* | 1.70±0.31* | 23.46±1.75* | 65.18±8.83* |
| COW | Control negative | 61.88±7.15 | 20.80±1.10 | 0.95±0.05 | 21.16±2.30 | 78.42±7.60 |
| COW | Case 1 | 63.50±5.05 | 20.65±0.48 | 1.13±0.26 | 20.50±1.98 | 75.28±9.25 |
| COW | Case 2 | 75.18±6.80* | 24.18±0.87 | 1.56±0.54* | 24.07±2.62* | 60.18±7.80* |
| COW | Case 3 | 100.22±4.90* | 26.05±1.08* | 1.42±0.63* | 23.33±1.80* | 66.03±4.96* |
| COW | Case 4 | 68.70±5.09* | 30.10±0.66* | 1.75±0.37* | 22.90±1.66* | 70.15±8.10* |
| COW | Case 5 | 98.52±7.70* | 28.80±2.50* | 1.50±0.16* | 24.80±2.01* | 69.05±5.90* |
| COW | Case 6 | 60.10±6.49 | 21.32±1.12 | 0.87±0.40 | 21.40±1.94 | 73.60±6.70 |
| COW | Case 7 | 87.00±5.34* | 29.89±0.49* | 1.66±0.65* | 22,90±2.30* | 58.10±4.15* |
| COW | Case 8 | 105.23±2.10* | 25.40±1.57* | 1.73±0.71* | 23.66±1.84* | 68.90±7.05* |
| COW | Case 9 | 95.41±5.87* | 27.09±1.26* | 1.51±0.44* | 25.01±2.43* | 55.80±6.43* |
| COW | Case 10 | 89.50±4.65* | 26.16±0.85* | 1.33±0.20* | 23.17±2.80* | 62.77±6.33* |
| Goats | Control negative | 85.13±7.80 | 22.69±1.06 | 1.52±0.35 | 18.56±1.66 | 79.18±5.62 |
| Goats | Case 1 | 95.20±3.37 | 25.66±1.20 | 1.68±0.50 | 19.93±0.98 | 75.80±5.80 |
| Goats | Case 2 | 121.65±6.15* | 33.90±2.17* | 2.05±0.81* | 22.40±1.22* | 69.15±7.07* |
| Goats | Case 3 | 123.70±6.46* | 36.80±2.04* | 1.97±0.75* | 23.25±1.70* | 62.77±6.61* |
| Ewes | Control negative | 67.10±5.02 | 20.80±0.80 | 1.30±0.09 | 20.05±2.26 | 58.79±5.98 |
| Ewes | Case 1 | 96.15±7.30* | 20.65±1.03* | 1.82±0.30* | 21.98±1.88* | 41.06±4.25* |
| Ewes | Case 2 | 101.73±4.83* | 24.18±1.10* | 1.71±0.25* | 23.53±1.03* | 45.82±5.62* |
| Ewes | Case 3 | 74.60±4.69 | 26.05±1.45* | 1.46±0.08* | 20.70±2.00 | 55.36±7.01 |

*Significant at P ? 0.05

Table (2) showed significant increase in the AST, ALT, Creatinine and Urea serum level. In contrast significant decrease in serum glucose level in most cases of examined bulls, cows, ewes and goats were recorded. Control negative means (Normal animal fed on silage free from AFB1)

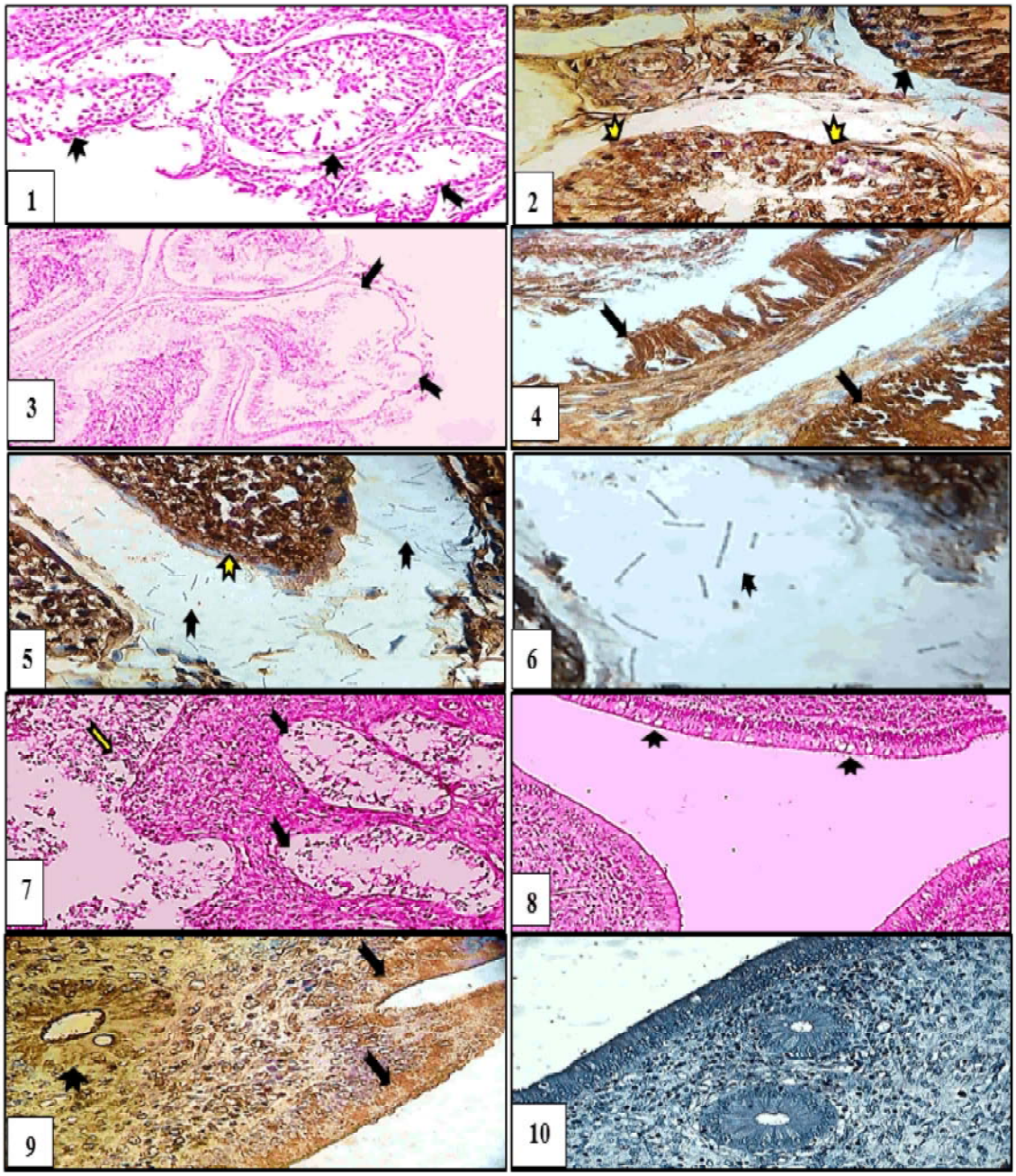


Plate 1: Histopathological and immunohistochemical Findings in the reproductive organs.

**Uterus:** Uterus in 7 over 10 cows showed severe ulcerative endometritis. Endometrial and uterine gland epithelium was severely degenerated, necrosed and desquamated in the lumen of glands. Clumps of necrotic debris in the uterine lumen associated with diffuse inflammatory cell infiltration in lamina propria were noticed (Fig. 7). Ewes and goats revealed mild vacuolar degeneration in endometrium and moderate infiltration of inflammatory cell in the lamina propria submucosa (Fig. 8). AFB1 antigen in uterus of 7 cows was moderately detected in endometrial epithelium and uterine glands (Fig. 9) in comparison to negative control of the same sample were showed (Fig. 10).

**Kidney and Liver:** Kidney in 9 cows, 3 bulls and 1 goat showed severe degeneration and necrosis in proximal and

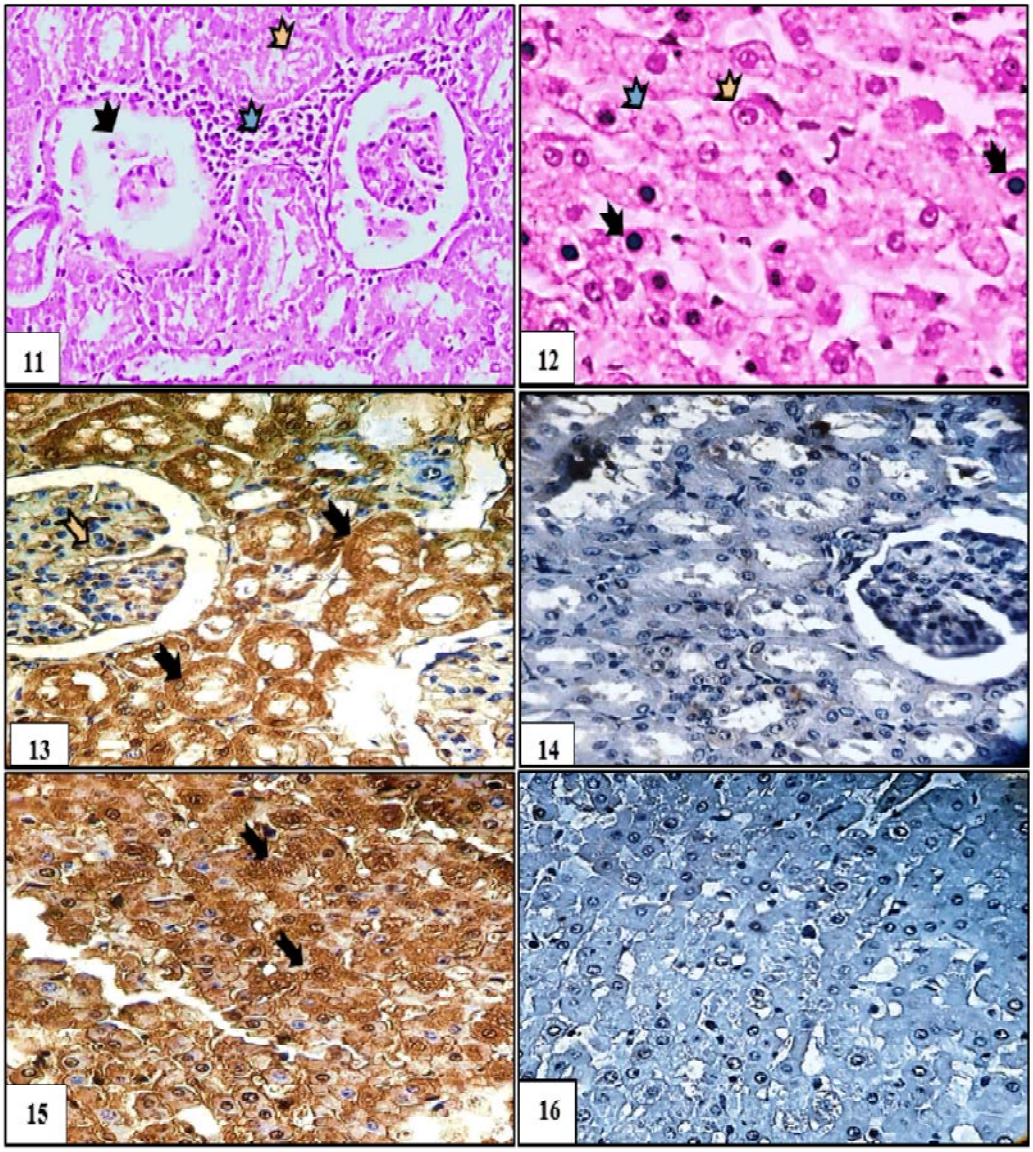


Plate 2: Histopathological and immunohistochemical Findings In kidney and liver.

distal convoluted tubules. Moderate multifocal peri glomerular infiltration of inflammatory cells mainly lymphocytes was found. Glomerular taft fragmentation was pronounced in some renal corpuscles (Fig. 11). Liver in 9 cows, 3 bulls and 1 goat was exhibited severe diffuse degeneration and necrosis of hepatocytes. Hyperchromatosis was diffusely pronounced in hepatocyte nuclei which appeared darkly blue stained nuclear material in comparison to normal and necrotic nuclei (Fig. 12). 9 cows, 4 bulls and 1 goat exhibited strong positive immunoreaction against AFB1antigen in renal tubules and mild reaction in glomerular taft (Fig. 13) as compared with negative control of the same sample was noticed (Fig. 14). AFB1 antigen was moderately detected in hepatocytes of 1 goat and strongly detected intracellularly in most of hepatocytes of 9 cows and 4 bulls (Fig. 15) as compared with negative control of the same sample (Fig. 16).

**Legands of Plates**

**Plate 1 Histopathological and Immunohistochemical Findings in the Reproductive Organs:** (Fig. 1) Testis of bull showed degeneration, necrosis and desquamation of spermatocytes into the lumen of seminefrous tubules (black arrows) x 100. (Fig. 2) Immunohistochemistry in testis of bull showed high intensity of AFB1 antigen in

most spermatocytes (black arrow) and in Sertoli cells (yellow arrows) × 200. (Fig. 3) Severe multifocal ulceration in the lining epithelium of bull epididymis was seen (black arrows) ×100. (Fig. 4) Epididymis of bull showed massive AFB1 antigen in epididymis (black arrows) × 400. (Fig. 5) *Mycotic hyphae* were found intertubular and exhibited negative immunoreaction as a blue coloration (black arrows) ×200 associated with High intensity of AFB1 antigen in most spermatocytes (yellow arrow) × 200. (Fig. 6) High magnification of fig. 5 exhibited clearly the *Mycotic hyphae* (black arrow) × 1000. (Fig. 7) Severe diffuse ulcerative endometritis in uterus of cows was observed (yellow arrow) accompanied with severe necrosis and desquamation in the lining epithelium of endometrium and uterine glands (black arrows) ×100. (Fig. 8) Mild vacuolar degeneration found in goat endometrium (black arrows) ×100. (Fig. 9) Intracytoplasmic AFB1 antigen was moderately noticed in endometrium of cow (black arrows) × 400. (Fig. 10) uterus of cow showed the negative control of the same sample of fig. 9 × 200.

**Plate 2: Histopathological and Immunohistochemical Findings in Kidney and Liver:** (Fig. 11) severe degeneration in most of renal tubules of goat was noticed (yellow arrow). Renal glomeruli fragmentation was found (black arrow) accompanied with moderate multifocal periglomerular infiltration of inflammatory cells mainly lymphocytes ×200. (Fig. 12) Hyperchromatosis in hepatocytes nuclei of cows was moderately observed (black arrow) as compared to normal nucleus (yellow arrow) associated with granular degeneration of hepatocytes (blue arrow) ×400. (Fig. 13) AFB1 antigen was detected intracytoplasmic in renal tubules of cow´s kidney (black arrows). Mild reaction in glomerular tuft was appeared (yellow arrow) × 200. (Fig. 14) kidney of cow showed negative control of the same sample of fig.15 × 200. (Fig. 15) Liver of cow showed massive diffuse Intracytoplasmic AFB1 antigen in most of hepatocytes (black arrows) × 400. (Fig. 16) liver of cow showed negative control of the same sample of fig. 15 × 400.

## DISCUSSION

Ruminants exposed to a multiple types of mycotoxin where it consume forages and feed byproducts more than dry grains [9]. Aflatoxin toxicity considered as safety and public health issues worldwide [19]. The maximum allowable level for AFB1 and total aflatoxins is 2 and 4 µg/kg respectively of nuts, peanuts, cereals and other products manufactured for direct intake [20]. In USA permissible level for total aflatoxins is 20µg/kg [21]. Turkish legal limits for AFB1 and total aflatoxins are 5 and 10 µg/kg respectively [22]. In this study we deal with a case of chronic aflatoxicosis in bull, cows, ewes and goats which consumed silage contaminated with aflatoxins for three month. Chronic aflatoxicosis in animals was causing impairment in reproductive efficiency as abortion, low birth weight and hormonal disturbances [8, 9]. AFB1 causes rise in liver enzymes as in table 2 that reflect into liver damage [6].

Histopathological findings in testis and epididymis of the 4 bulls revealed severe degeneration and necrosis in the spermatocytes. Multifocal desquamation of the epididymal epithelium was observed. These finding come in agreement with Agnes and Akbarsha [23] who reported significant reduction in sperm concentration in mice injected with aflatoxin. Leydig cells have been hypertrophied and vacuolated due to toxicities, that leading to inhibition of testosterone production which controlling the spermatozoa production in testis. Decreased the counts and motility of sperm were also reported [24].Ulcerative endometritis were paralleled with the fact that; Aflatoxicosis in cow caused abortion and reduced fertility [25]. In human aflatoxin could be crossed the placenta meanwhile transported to fetus and cause detrimental effects [26].

Aflatoxins have been reported to cause liver cirrhosis and liver cancer [3]. Severe degeneration and necrosis of most of hepatocytes could be explained by the fact that; AFB1 in liver and kidney forming adducts with DNA, RNA and proteins so protein biosynthesis impaired and causes degranulation of endoplasmic reticulum [10]. AFB1 causes liver failure so become unable to detoxify ammonia of protein metabolism leading to hyperammonemia that pass through the blood brain barrier leading to increase synthesis of glutamate neurotransmitters hence brain cell damage occure [7].

Hyperchromatosis existence in liver as a sign of carcinogenicity, can be discovered as; CYP450 enzyme metabolizes AFB1 to form 8, 9- epoxide [27]. Epoxide has high affinity to binds with guanine bases in DNA to form afltoxin-N7- guanine [28]. That in turn substitute guanine instead of thymine causing transversion mutation hence affecting on p53 suppressor gene so hepatocellular carcinoma (HCC) occure [6].

Several degenerative and necrotic changes in our study could be explained by hypoxia and energy deficiency due to mitochondrial function loss. Reactive aflatoxin-8, 9-epoxide preferentially binds to mitochondrial DNA that hinders ATP production and FAD/NAD-linked enzymatic functions so causing cell aging mechanisms [6].

AFB1 antigen was strongly observed in Sertoli cells and spermatocytes of bulls; also found intracellular in endometrium of cows. That confirms the ability of AFB1 to gain access intracellularly to Sertoli cells, spermatocytes and endometrium. Immunohistochemistry could be used to monitor AFB1exposure by measurement of DNA adducts in liver. Although these methods may not be useful for routine monitoring of healthy individuals, they are an important tool to the role of AFB1 in HCC [29].

## CONCLUSIONS

The main conclusions of study; Bad stored silage created the conditions for asperagillus flavus growth. AFB1 caused clinicopathological and histopathological changes. AFB1 antigen was detected by IHC in the reproductive system of cattle. Sheep and goats showed high resistance to aflatoxin.

## ACKNOWLEDGMENT

Authors contribute special regards to the Central Lab of Mycotoxins in National Research Center for the effort of team work in HPLC measurement for all Aflatoxin fractions in spoiled silage samples.

## REFERENCES

1. Magan, N.D. and D. Aldred, 2007. Post-Harvest Control Strategies Minimizing Mycotoxins in the Food Chain. International Journal of Food Microbiology, 119: 131-139.
2. Cortés, G., M. Carvajal, I. Méndez-Ramírez, E. Avila-González, N. Chilpa-Galván, P. Castillo-Urueta and C.M. Flores, 2010. Identification and quantification of aflatoxins and aflatoxicol from poultry feed and their recovery in poultry litter. Poultry Science, 89: 993-1001.
3. Thrasher, J.D., 2012. Aflatoxicosis in animals. Aflatoxins and Health, www.alphaboostjuice. com/AFLATOXICOSIS_IN_ ANIMALS.pdf.
4. Traverso, A., V. Bassoli, A. Cioe, S. Anselmo and M. Ferro, 2010. Assessment of aflatoxin exposure of laboratory worker during food contamination analyses. Assessment of the procedures adopted by an A.R.P.A.L. Laboratory (Liguria Region Environmental Protection Agency). Med. Lav., 101: 375-380.
5. Kensler, T.W., B.D. Roebuck, G.N. Wogan and J.D. Groopman, 2011. Aflatoxin: a 50- year odyssey of mechanistic and translational toxicology. Toxicology Science, 120: S28-S48.
6. Bbosa, G.S., D.K.A. Lubega, J. Ogwal-Okeng, W.W. Anokbonggo and D.B. Kyegombe, 2013. Review of the Biological and Health Effects of Aflatoxins on Body Organs and Body Systems. Aflatoxins - Recent Advances and Future Prospects, pp: 239-265.
7. Hussein, Z., M.Z. Khan and Z.U. Hassan, 2008. Production of aflatoxins from Aspergillus flavus and acute aflatoxicosis in young broiler chicks. Pakistan Journal of Agricutlural Sciences., 45: 95-102.
8. Hussein, H.S. and J.M. Brasel, 2001. Toxicity, metabolism and impact of mycotoxins on humans and animals. Toxicology Journal, 167: 101-134.
9. Ewuolaa, E.O., O.A. Jimohb, A.D. Belloc and A.O. Bolarinwaa, 2014. Testicular biochemicals, sperm reserves and daily sperm production of West African dwarf bucks fed varied levels of dietary aflatoxin. Animal Reproduction Science, 148: 182-187.
10. Wangikar, P.B., P. Dwivedi, N. Sinha, A.K. Sharma and A.G. Telang, 2005. Effects of aflatoxin B1 on embryo fetal development in rabbits. Food Chemical Toxicology, 43: 607-15.
11. Prabu, P.C., P. Dwivedi and A.K. Sharma, 2013. Toxicopathological studies on the effects of aflatoxin B1, ochratoxin A and their interaction in New Zealand White rabbits. Experimental Toxicological Pathology, 65: 277-286.
12. Truckess, M.W., M.W. Stack, S. Nesheim, S.W. Page, R.H. Albert, T.J. Hansen and K.F. Donahue, 1991. Analytical Chemistry, Immunoaffinity column coupled with solution fluorometry or liquid chromatography post column Derivatization for determination of alfatoxins collaborative study. Journal of the Official Association, 74: 81-88.
13. Trinder, P., 1961. Enzymatic colorimetric method for glucose determination. Annual Clinical Biochemistry, 6: 24-39.

14. Ritman, S. and S. Frankle, 1957. Glutamic-Pyruvate Transaminase assay by colorimetric method. American Journal of Clinical Pathology, 28: 56.
15. Oliver, I.T., 1955. A Spectrophotometric Method for Determination of Creatinine Phosphokinase and Myokinase. Biochemistry Journal, 61: 116-120.
16. SAS, 2004. Statistical Analysis Systems. Version 9.2. SAS institute Cary, NC.
17. Bancroft, J.D., A. Stevenes and D.R. Turner, 1996. Theory and practice of histological technique. 4th ed. Churchill Livingstone Inc New York, Edinburgh, London, Melbourne, San Francisco, Tokyo.
18. Haines, D.M. and E.G. Clark, 1991. Enzyme immunohistochemical staining of formalin-fixed tissues for diagnosis in veterinary pathology. Canadian Veterinary Journal, 32: 295-302.
19. Atanda, O., H.A. Makun, I.M. Ogara, M. Edema, K. Idahor, M.E. Eshiett and B.F. Oluwabamiwo, 2013. Fungal and mycotoxin contamination of Nigerian foods and feeds. In. Mycotoxin and food safety in developing countries. Chapter, 1: 1-38.
20. Commission of the European Communities, 2001. Setting maximum levels for certain contaminants in food stuffs. Commission Regulation (EC) No.: 466, L 77: 1-13.
21. Creppy, E.E., 2002. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicology Letters, 127: 19-28.
22. Turkish Food Codex, 2002. Türk Gıda Kodeksi Gıda Maddelerinde Belirli bulaþanların maksimum seviyelerinin belirlenmesi hakkinda tebliğ. (Legislation about determination of maximum levels of certain contaminant in foods.) Resmi Gazete, 23 Eylül 2002, Sayı 24885. Ankara: Baþbakanlık Basımevi.
23. Agnes, V.F. and M.A. Akbarsha, 2003. Spermatotoxic effect of aflatoxin B1 in the albino mouse. Food and Chemical Toxicology, 41: 119-130.
24. Whitlow, L.W.W., M.J. Hagler and D.E. Diaz, 2010. Mycotoxins in feeds. Feed Quality Mycotoxins, pdf., pp: 74-84.
25. AAFRD Alberta Agricultural, Food and Rural Development (AAFRD), 2003. Moldy Feed and Reproductive Failure in Cows. http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex849/$file/666-5. pdf.
26. Ibeh, I.N., D.K. Saxena and N. Uraih, 2000. Toxicity of aflatoxin: effects on spermatozoa, oocytes and *in vitro* fertilization. Journal of Environment, Pathology, Toxicology and Oncology, 19: 357-361.
27. Kitada, M., M. Taneda, H. Ohi, M. Komori, K. Itahashi, M. Nagao and T. Kamataki, 1989. Mutagenic activation of aflatoxin B1 by 450 HFLa in human fetal livers. Mutation Research, 227: 53-58.
28. Guengerich, F.P., 2001. Forging the links between metabolism and carcinogenesis. Mutation Research, 488: 195-209.
29. Bechtel, D.H., 1989. Molecular dosimetry of hepatic aflatoxin Bl-DNA adducts: linear correlation with hepatic cancer risk. Regular Toxicological Pharmacology, 10: 74-81.