RESEARCH ARTICLE

Histopathological and Immunohistochemical Study of Aflatoxin B1 in Freshly Slaughtered Iraqi Sheep Meat, using CD Marker of TNF-α

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ABSTRACT

The current study was conducted in Baghdad governorate (Karkh and Al-Rasafa regions) which included collecting 50 samples of freshly slaughtered sheep meat randomly collected from local slaughter areas and approved governmental slaughterhouses (25 liver and 25 ulna muscles). The results of the aflatoxin B1 detection showed that all samples were contaminated with this toxin at different concentrations ranging from 25–422 ppb and 65–492 ppb for each ulna muscles and liver, respectively. The histopathological and immunological study was conducted in meat samples containing higher and lower concentrations of the toxin. The results of the pathological study in the liver revealed that the concentration (492 ppb) caused thickening of the nuclei, water degeneration and necrosis, but the concentration (467 ppb) caused several damages represented by thickening of the nuclei and an increase in the number of kepffer cells. In contrast, no obvious damages was observed at the concentration (65 ppb).

In the muscles, the results showed that the concentrations (422 and 384 ppb) caused tissue damage represented by the homogeneous acid aggregation, while no damage was found at the concentration 25 ppb. The findings of the immunohistochemical study showed the same concentrations for both liver and muscle samples using CD marker of TNF-α; in the liver, at the concentration (492 ppb), there was strong immune expression (+++), while the immune expression was (++) at the concentration (467 ppb), and no expression (-) was detected at the concentration (65 ppb). In addition, the results in the muscles samples showed that samples that contain (422 ppb), the immune expression was (+++), while the expression at the concentration (384 ppb) was (++), whereas no expression was seen at the concentration (25 ppb).

Keywords: Aflatoxin B1, Histopathology, Immunohistochemistry, TNF-α.

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INTRODUCTION

Aflatoxins are secondary metabolites naturally produced by some species of *Aspergillus* such as *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. ochraceoroseus*, *A. australis* and *A. psudotamarii*.[1] One of the characteristics of aflatoxins is colourless, odorless, and tasteless, so it is not easy to detect them with the naked eye.[2] Additionally, more than 20 types of aflatoxins have been known. However, the most common and toxic aflatoxins that have been identified are B1, B2, G1, G2.[3] B and G refer to the brilliance of the colors when separated on the chromatographic plates, thus when exposed to ultraviolet rays, one of them gives blue shin and the other give green shine, whereas the numbers 2–1 refer to the transfer factor (RF) caused by spots on the chromatographic plates.[4]

Interestingly, Aflatoxin B1 is the most toxic, as its presence causes great economic losses in the field of agriculture, industry and poultry farming, as it increases fatalities and has harmful health effects for humans and animals.[5] Field crops contamination with aflatoxin increases with stress on the plant, while after harvesting, the pollution increases due to the conditions causing or stimulating the growth of fungi during the storage period. The most important factor is the concentration of moisture content and relative humidity of the medium and the presence of rodents or insects serving as transfer of fungi and producing toxins. Moreover, due to the high prevalence of aflatoxin-producing fungi in food products, International Atomic Energy Agency (IAEA) organization currently allows 20 ppb of aflatoxin in foodstuffs as the maximum allowable limit.[6] The infection of animals with mycotoxins depends on environmental factors, including the climatic conditions, soil, management, genetic factors represented by type, strain and class, and physiological factors such as nutrition, age and others.[7]

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Mycotoxins are secreted in many agricultural crops, and grains are the most important of those crops in which the level of contamination was studied because they represent an important food for humans and animals.[8] Furthermore, toxins are found in food and feed due to a complex series of interactions between the causative fungi and contaminated products, various environmental factors, and the host.[9]

Aflatoxin b1 is considered one of the most dangerous compounds due to its presence in foodstuffs and feedstuffs, and it has obvious effects on human and animal health, as it affects the digestive, urinary and reproductive systems, neurological disorders, as well as cancer cases and genetic mutations.[10] Moreover, in humans Aflatoxin b1 may lead to tumors, hemorrhage, distortions and general weakness, as well as its effects on animals through decreasing the productivity and many viral and bacterial diseases as a result of destroying the immune system.[11] In terms of genetics, aflatoxin affects the molecular level due to its role in embryonic teratogens and genetic mutations, inhibiting the DNA and RNA construction, reproduction process and thus inhibiting the proteins biosynthesis.[12] It also affects the process of lipid metabolism, reduces the entry of oxygen into mitochondria, and then affects the rate of cell respiration, leading to the accumulation of fats in the liver, causing fatty liver disease.[13] However, the main target organ by aflatoxin is the liver, which causes cancer and cirrhosis in poultry, rodents, fish and mammal, and the disease resulting from exposure to aflatoxin can be classified as aflatoxin poisoning and is described as having an acute and chronic effect.[14]

Tumour necrosis factor alpha (TNF-α) is a potent pleiotropic cytokine produced by different types of lymphocytes,[15] such as NK cells in addition to macrophages, fibroblasts, neutrophils, osteoblasts, and Kupffer cells. Kupffer cell and smooth muscle cell.[16] TNF-α contains two cell surface receptors: TNFR1 and TNFR2. TNFR1 is characterized by its absolute presence in all cells in mammals, while TNFR2 is found in endothelial and immune cells. Both TNFR1 and TNFR2 restrict or modulate TNF-α activity.[17]

TNF-α has multiple functions; it regulates a variety of signaling pathways involved in immune inflammation and programmed cell death as it does not act as a major mediator in hepatocyte apoptosis that leads to liver damage but rather plays an important role in cellular proliferation, which leads to the regenerate, or the formation of liver cells. Additionally, TNF-α indirectly contributes to carcinogenesis through many inflammatory diseases such as fatty liver disease and chronic viral hepatitis; it helps infiltrate the inflammatory cells of tumors, promote angiogenesis, migration and invasion of tumor cells. These effects can be explained by the diverse cellular responses that TNF-α can exhibit.[18]

MATERIAL AND METHODS

Samples were collected from local slaughter areas and approved governmental slaughterhouses in Baghdad Governorate (Al-Karkh and Al-Rasafa regions), which included 50 samples of freshly slaughtered fresh sheep meat (25 livers and 25 ulna muscles) for a period from 5/10/2017 to 20/10/2017

Detection of Aflatoxin B1

Aflatoxin B1 was detected using enzyme-linked immunoassay (ELISA) technique according to the manufacturer's instructions (Qucking Biotech), where the samples were extracted and mixed with the conjugation enzyme and anti-toxin antibodies in an immunoassay dish, then the substrate A, and B was added. The absorbance was recorded using ELISA reader at wavelength of 450 nm, while the concentration was determined using standard curve based on the following equation:[19]

Percentage of absorbance value = (B/BO) × 100

B = Absorption standard for standard solutions or sample

BO = absorbance of blank solution

Histological Preparation

Samples were fixed in formalin (10%) to prepare these specimens for the histological study. The samples were passed with an ascending series of alcohols (70, 80, 90 and 100%); then all the samples are cleared using xylene and embedded in paraffin wax blocks. The paraffin wax blocks were cut by using microtome to prepare sections with 6 µm in thickness; the sections were stained using Haris Haematoxylin and Eosin stain.[20] The immune reaction was determined by the appearance or absence of dye in the tissue, symbolized by (+) in case of an immune reaction and the appearance of brown color in the cytoplasm. At the same time, the absence of dye is symbolized by the symbol (-) by using CD marker of TNF-α. The slides were then examined under a photographed microscope connected to a digital camera used to take pictures for the selected sections.

RESULTS AND DISCUSSION

The Histopathological Study

The results of the histopathological study in the liver, which contains the highest concentration (492 ppb), showed thickening in the nuclei, water degeneration, and necrosis (Figure 1). In comparison, in the concentration (467 ppb),

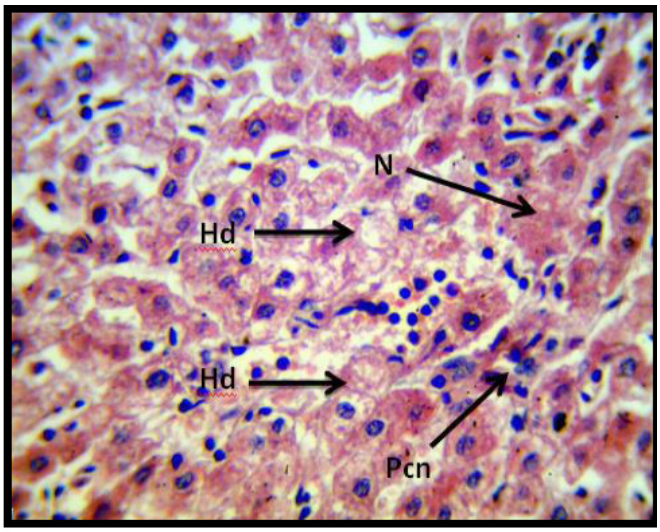

Figure 1: A section in sheep liver at concentration (492 ppb) of aflatoxin B1 showing the occurrence of thickening of the nuclei (Pen), hydrodegeneration (Hd) and necrosis (N), stain (H&E) (400X),

there were tissue damages represented by thickening of the nuclei and an increase in the number of kepffer cells (Figure 2). Whereas at the concentration of (65 ppb), there were no obvious damages (Figure 3), and this agrees with a study[21] on mice dosed with toxin at a concentration of 4 g of corn contaminated with toxin after 20 days of treatment and obvious damages were detected in the liver tissues represented by vascular congestion. In addition,[22] that the exposure of mice with different concentrations (44 ppb, 442 pp, 663 ppb) of toxin and after 5 days of treatment, it was found that high concentrations of the toxin caused degeneration of the nuclei, necrosis and fibrosis in the liver. Furthermore, another research[23] indicated that high concentrations of the toxin (20, 40, 60, 80 and 100 ppb) in mice for eight days caused degeneration in hepatocytes, severe hydrocyst and necrosis; this damage was severe with increasing the dose. Another research[24] studied the camel livers collected randomly from different slaughterhouses in Saudi Arabia and observed that the samples containing concentrations below the permissible limit did not notice any satisfactory changes in them. In contrast, those containing medium concentrations of toxin had cirrhosis, fatty clotting and water degeneration in the liver. As for the samples that contained high concentrations of aflatoxin B1, severe damages were observed, represented by gastroenteritis, cholangitis and cirrhosis of the liver.

The reason for the thickening of the nucleus is may be due to the effect of aflatoxin B1 on the nuclear material, causing abnormal changes in it, as it is known that aflatoxin B1 is one of the toxins that have a role in causing genetic mutation inside the living cells, then affecting the protein biosynthesis process, as we as the toxin affects the plasma membrane and then loss of the selective permeability property, which leads to the exit the water outside the cells, whereas the liver necrosis, it may occur as a result of cell degeneration due to an imbalance in the fat transfer process (Mclean and Dutton, 1995) or fat accumulation[25] as a result of the metabolism process and the close relationship with the digestive system. Thus liver is susceptible to infection with various types of toxins and other pathogens by 75%, and the blood from the digestive system reaches directly to the liver and then to the spleen through the portal veins, which brings toxins in a diluted form. There are many mechanisms responsible for triggering liver injury and exacerbating the damage process. Interestingly, many toxins and chemicals damage mitochondria that produce energy, which leads to the release of double amount of oxidants that infect hepatocytes. Activating some enzymes in the cytochrome p450 system such as cyp2e1, causes oxidative stress[26] and injury in hepatocytes and bile duct cells leads to the accumulation of bile acid, which enhances the fibrosis process in the liver.[27]

On the other hand, the results in the muscles showed that the concentration (422 ppb and 384 ppb) caused tissue damage represented by the accumulation of homogeneous acid (Figures 4 and 5), while at the concentration 25 ppb, no tissue damage was appeared (Figure 6). The high concentrations of the toxin have a role in the deterioration of skeletal muscle,[28] and in a previous study conducted on rabbits, they were treated with aflatoxin B1 at a concentration of 2 ppm for 4 months. After an autopsy, it was found that the toxin in the muscles exceeded the permissible limits with the presence of pathological changes,[29] While in mice treated with 200 mg/kg of toxin for 30 days, it was found that aflatoxin B1 has a role in skeletal muscle degeneration, damage of fibroblasts, connective tissue and muscle fibers.[30] While there is a high percentage of toxins stored[31] in the cardiac muscles of sheep and caused muscle necrosis, mononuclear cell inflammation, and hemorrhage in the area between the heart and the muscles.

The basic unit of the skeletal skeleton is the muscle fiber, a flexible protein that carries out the process of contraction, and these fibers contain an endoplasmic reticulum that differs from those in normal cells that store calcium over time of need (muscle contraction). The concentration of calcium inside the

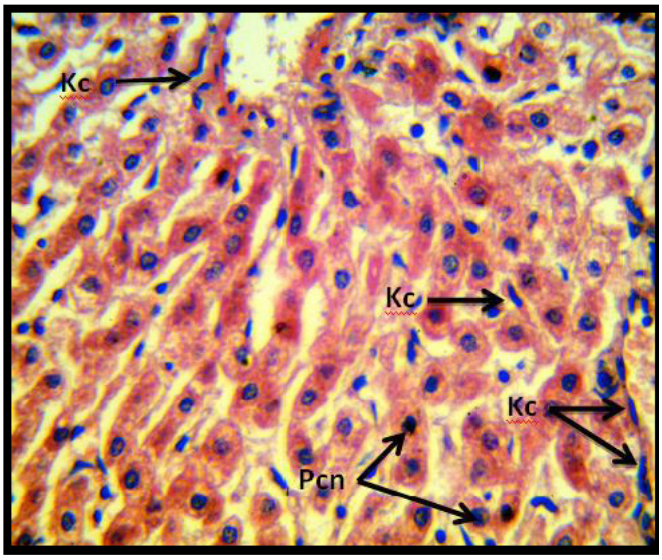

Figure 2: A section in sheep liver at concentration (467 ppb) of aflatoxin B1 showing an increase in the thickening of nuclei (Pen) and the number of Kupffer cells (Kc).Stain (H&E) (400X).

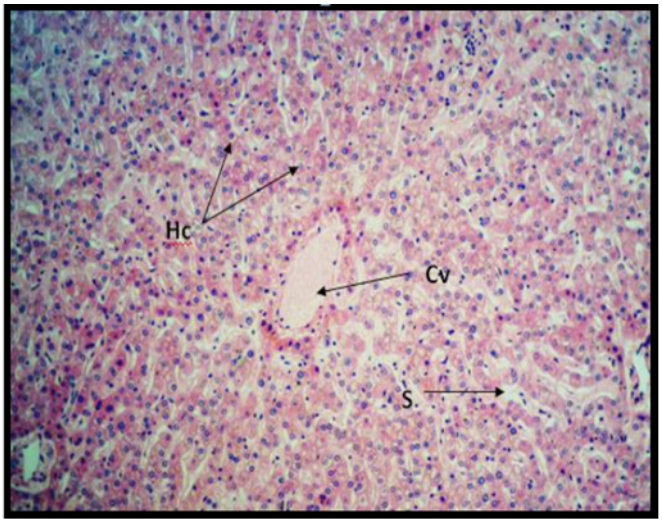

Figure 3: A section in sheep liver at concentration (65 ppb) of aflatoxin Bl showing the histological structure of the liver central vein (cv), hepatocytes (HC) and blood simsoids (S), Stain (H&E) (100X).

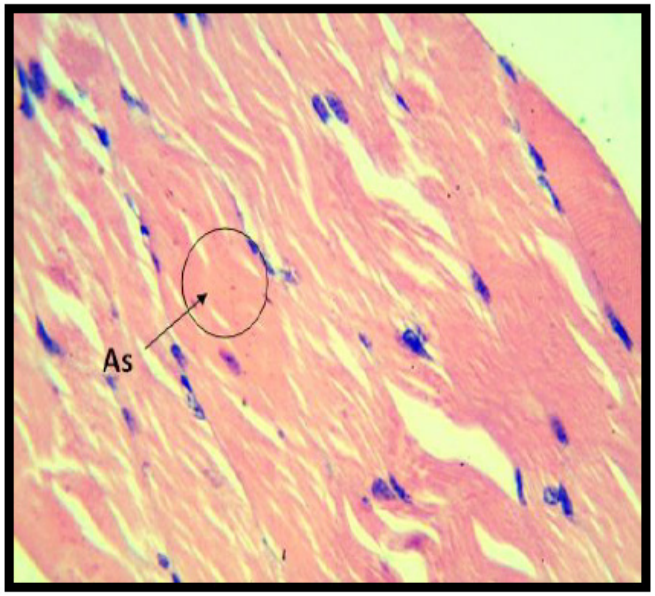

Figure 4: A section in sheep mescle at concentration (422 ppb) of aflatoxin B1 showing a region of homogeneous acidic cytoplasm (As). Stain (H&E) (400X).

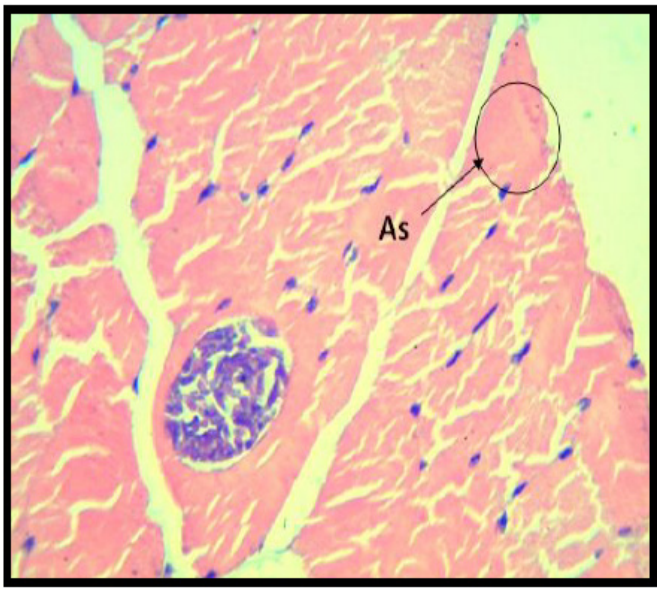

Figure 5: A section in sheep muscle at concentration (384 ppb) of aflatoxin B1 showing a region of homogeneous acidic cytoplasm (As). Stain (H&E) (400X).

cells is low, and after exposure to toxins, or various chemicals, this may lead to the release a high amount of calcium, which causes contraction by T cells, which are transverse tubules whose function is the transmission of nerve impulses to the inside of the muscle that contributes to the muscle response.[29] Furthermore, a study confirmed that Aflatoxin B1[30] affects muscle, causing an increase in chromatin density, and an increase in satellite cells. The rise in these cells is due to aflatoxin B1, which stimulates them to multiply in response to an injury to renew the muscles or causes blood vessel necrosis. Necrosis occurs because of a deficit, or decrease in O_2 exchange

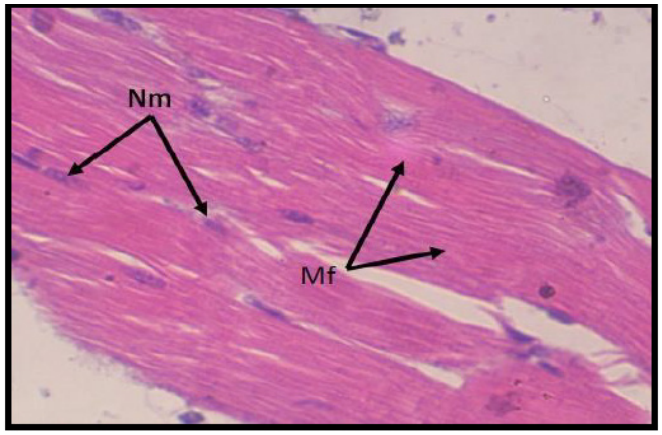

Figure 6: A section in sheep muscle at concentration (25 ppb) of aflatoxin B1 showing the muscular fibers (MF) and the nuclei of the muscular fibers (Nm). Stain (H&E) (400x).

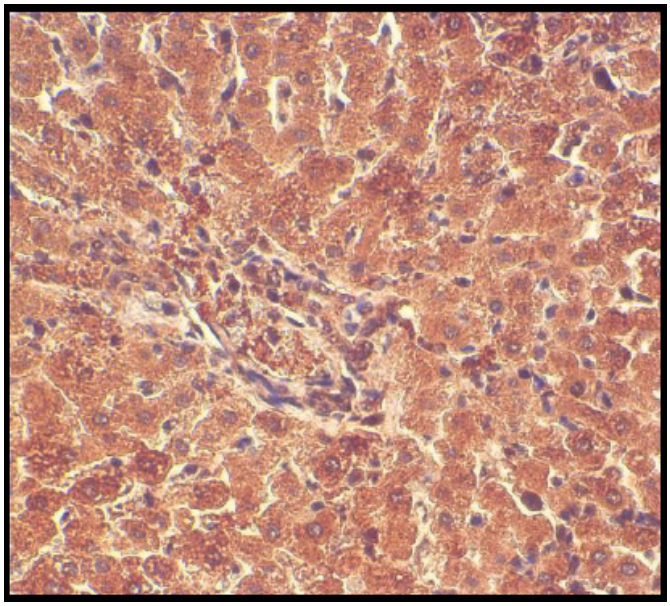

Figure 7: A section in sheep liver at a concentration of (492 ppb) of aflatoxin B1 showing the intensity of the immune reaction in the tissue (--+), CD marker of (TNF-a) (400x)

within the muscles, and the skeletal muscle due to damage or a decrease in the number of capillaries

The Immunohistochemistry Study

The results of the immunohistochemistry study using CD marker of TNF-α in the liver found that toxin at a concentration of 492 ppb caused strong immune expression (+++) (Figure 7). While the immune expression was (++) at concentration 467 ppb (Figure 8), and there was no expression (-) at concentration 65 ppb (Figure 9). In the muscles, the results showed that the immune expression was (+++) in the samples containing a concentration of 422 ppb (Figure 10), whereas it was (++) at a concentration 384 ppb of toxin (Figure 11); however, there was no expression in the concentration of 25 ppb (Figure 12).

The findings of immunohistochemistry study indicated that aflatoxin B1 binds with TNF-α during the cellular inflammation and general liver damages[32] where aflatoxin B1

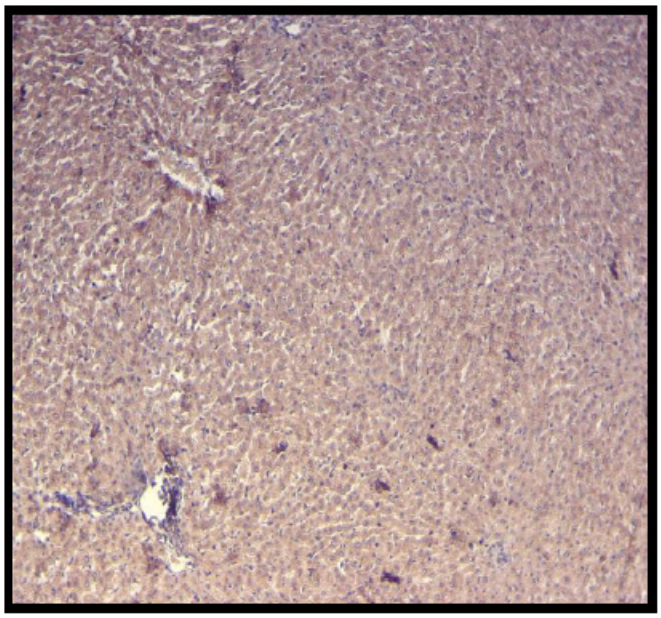

Figure 8: A section in sheep liver at a concentration of (467 ppb) of aflatoxin Bl showing the intensity of the immune reaction in the tissue (+), CD marker of (TNF-a) (400x)

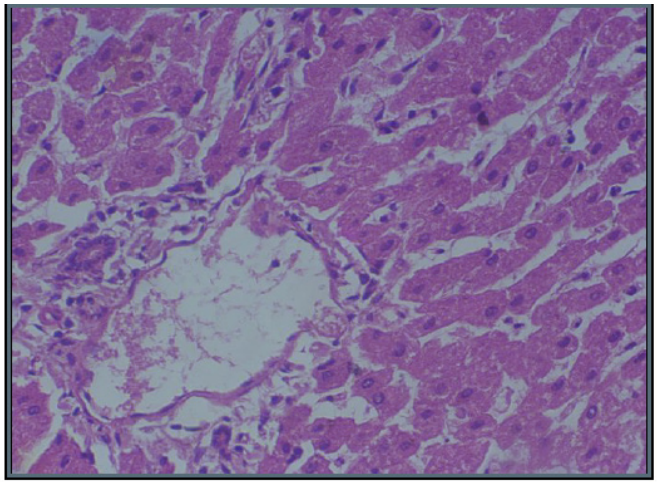

Figure 9: A section sheeper of concentration of 165 ppbl odatletom B1 showing the absence of the instruction in the tissus, CD maker of (INF0) (400X

causes an overproduction of TNF-α and this is in agreement with a study[33] that observed the treatment of rats with toxin at different concentrations (385, 867, 1807 mg/kg), a strong immune expression was found in the spleen using CD marker of TNF-α. Moreover,[32] study on mice treated with toxin at a concentration of (100 ppb), it was found that the higher immune expression of TNF-α was associated with higher exposure to the toxin in the liver, and this may due to the effect on the lymphocytes which important in the regulation of immunity and inflammation.[34] Additionally, the exposure of rats to low concentrations of aflatoxin B1 (5-75 ppb) for a long time, plays a role in chronic inflammation by regulating cytokine expression and increasing TNF-α production.[35] The production of these cytokines includes in the pathological events represented by necrosis and fibrosis, as it works to repair in case of exposure

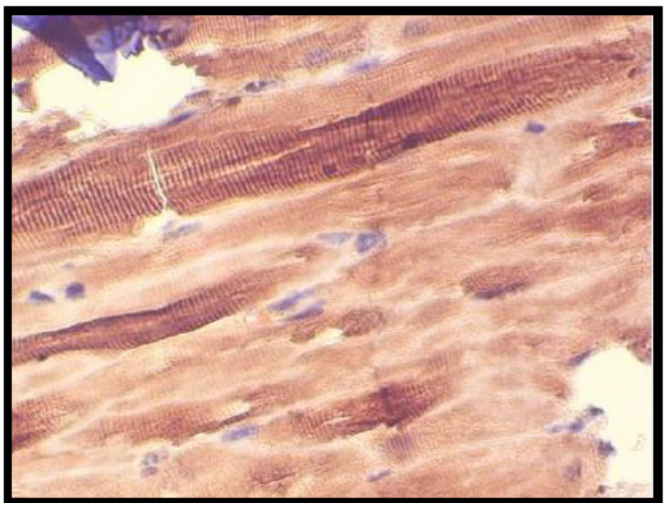

Figure 10: A section in the ulan muscle of sleep at a concentration of (422 ppb) of aflatoxin B1 showing the intensity of the main reaction in the tissue (+++), CD marker of (TNF-a) (400X)

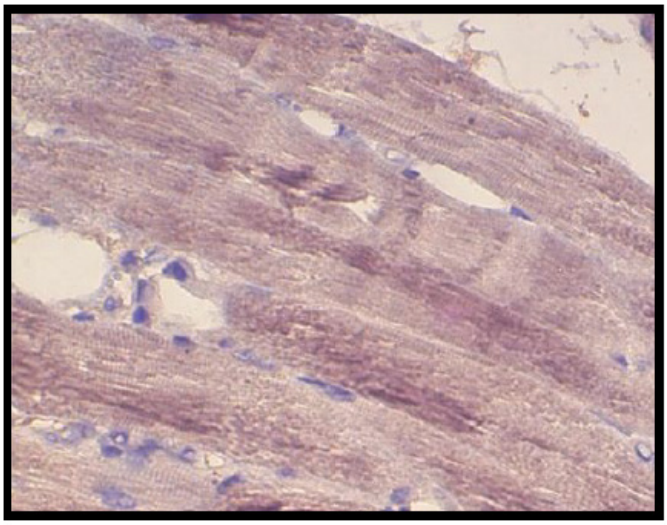

Figure 11: A Section in the ulna mascle of sheep at a concentration of (384 ppb) of aflatoxin B1 slowing the nitensity of the immune reaction in the tissue (-), CD marker of (TNF-a) (400X)

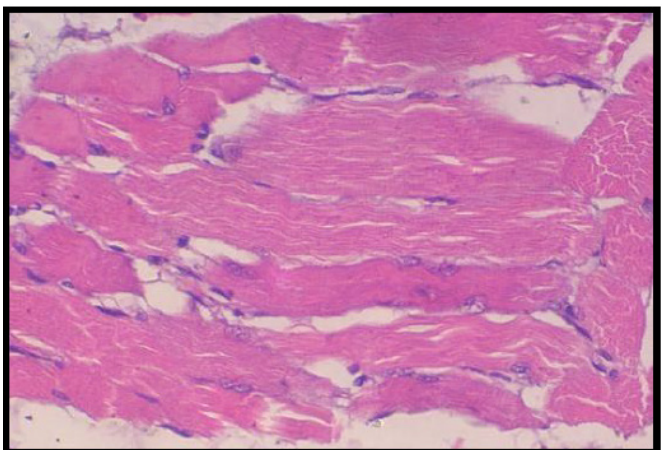

Figure 12: A section in the ulna muscle of sheep at a concentration of (25 ppb) of aflatoxin B1 showing the absence of the immune reaction in the tissue, CD marker of (TNF-α) (400X)

to different types of toxins.[36] Cytokines such as TNF-α play a role in many inflammations, including hepatotoxicity, and other damages that cause necrosis, as they are released by kupffer cells, considering that 80% of macrophages are present in the liver and these cells are the source of inflammatory cytokines, and the over secretion of these cytokines results in an immune response represented by an increase in LPS production, which affects liver functions such as metabolism and then liver failure.[37]

Initially, TNF-α works to repair damaged and necrotic tissues in the damaged organ in response to the necrosis process, which works together with endothelial cells and epithelial cells. TNF-α also is the major regulator that helps in tissue repair through programmed cell death and proliferation and initiation of the inflammatory process, as it works to recruit and activate lymphocytes such as neutrophils and monocytes in the injured site; the cell damage increases TNF-α levels that lead to an increase in macrophage activation at the site of damage.[37]

In the muscles, when aflatoxin B1 is present at high concentration in the liver, part of toxin is stored in the muscles, but in small quantities than in the liver, and this is consistent with other observations,[38] study using chickens fed with toxin (50 ppb, 100 ppb) for six weeks. After the autopsy and analyzing the liver and muscle tissues, it was found that there were high levels of the toxin in the liver and in the muscles, but at a lower concentration than in the liver. Furthermore, other[39] study on birds found that the concentrations of the toxin in the liver were in the range of 10.14–13.83 ng/g, while in the muscles, the concentration was 0.97–1.83 ng/g.

CONCLUSION

TNF-α is a polypeptide cytokine that has been associated to muscle pathogenesis, however, its effect on skeletal muscle is largely unknown; it stimulates muscle growth, apoptosis, while it is the effect on muscle through an effect on its receptors which then stimulate a cellular response.[40] It also has a role in activating satellite cells in muscles[41] and its secretion is increased by mast cells, as well as by macrophages and neutrophils, which accumulate rapidly in the injured area, where its secretion increases in the damaged muscle fibers and is expressed by myoblasts.[42]

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