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Effect of Immunization against Aflatoxin B1 on the Expression of COX-2 in Liver, Kidney and spleen of Rabbits treated with aflatoxin B1

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Abstract
Aflatoxin B1 is a mycotoxin produced mainly by the fungus *Aspergillus flavus* and *A. parasiticus* in food and feed. It is considered as a carcinogenic toxin for human and animals. The current study was designed to investigate the expression of COX-2 in liver, kidney and spleen of immunized and non-immunized rabbits against aflatoxin B1 which treated with aflatoxin B1. Immunohistochemical results indicated that COX-2 highly expressed in the liver, kidney and spleen (4.00±0.00*, 3.66±0.33*, 2.00±0.00* respectively) (P≤ 0.05) of aflatoxin B1-treated rabbits (0.7 mg/kg) and their expression was significantly reduced by immunization of rabbits against aflatoxin B1 using prepared immunogen consisted of (aflatoxin B1- bovine serum Albumin Conjugate using concentration 100 µg and 200 µg), and the expression was reduced using concentration 100 µg more than 200 µg.
Keywords: Aflatoxin B1, Antibody, Immunization, COX-2

1. Introduction

Aflatoxin B1 is toxic secondary metabolites which produce by some fungi include *Aspergillus flavus* and *Aspergillus parasiticus* in foods and feeds, it has been associated with various diseases, such as aflatoxicosis, in livestock, domestic animals and humans throughout the world. The occurrence of aflatoxin B1 is affected by some environmental factors such as geographic location, agricultural and agronomic behavior, and the susceptibility of commodities to fungal invasion during pre-harvest, storage, and processing periods. Aflatoxin B1 has received greater interest than any other mycotoxins due to of their carcinogenic effect in laboratory animals and their acute toxicological effects in humans (Eaton and Groopman, 1994).

Aflatoxin B1 is low molecular-weight secondary fungal metabolite, it was devoid of antigenicity, and hence it must be conjugated to suitable carrier (such as bovine serum albumin). Efforts to improve antibody production against aflatoxin B1 were made by immunization of lab animals with aflatoxin B1 –BSA Conjugates (Chu and Ueno, 1977).

Vaccination against aflatoxin B1 is able to induce antibodies which reduce the toxic effects of aflatoxin B1 (Langone and Van Vunakis, 1976).

Cyclooxygenase-2 is an enzyme involved in inflammatory processes and a rate limiting enzyme in prostaglandin biosynthesis from arachidonic acid. Inappropriate up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues (Mohan, and Epstein, 2003).

2. Materials and Methods:

2.1 Preparation of Aflatoxin B1 Carboxymethyl oxime:

Aflatoxin B1 Carboxymethyl oxime was prepared according to the method described by (Nanju *et al*., 2004).

2.2 Preparation of Aflatoxin B1 – BSA Conjugate:

The conjugate was prepared according to the method described by (Chu and Ueno, 1977).

2.3 Antibody production against Aflatoxin B1:

2.3.1 Preparation of injecting solutions:

A- The first injection solution was consisted of 1mg of BSA dissolved in 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio, and the final result was very dense emulsion.
B- The second injection solution was made by adding of 100 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio.
C- The third injection solution was made by adding of 200 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with complete Freund adjuvant (1:1) ratio.
D- The fourth injection solution was made by adding of 100 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl (0.9%) which mixed with incomplete Freund adjuvant (1:1) ratio.
E- The fifth injection solution was made by adding of 200 µg of Aflatoxin B1- BSA Conjugate to 1 ml NaCl

(0.9%) which mixed with incomplete Freund adjuvant (1:1) ratio.

2.3.2 Immunization schedule:

For Immunization the multiple – site intradermal method (Nieschlag *et al*., 1975) was followed with some modification, New Zealand White rabbits were immunized in their back using the following schedule (Two groups of New Zealand White rabbits ((3 rabbit for each concentration)) were used):

• Week (Zero): Two milliliter of solution (A) was injected as priming dose for the both groups of rabbits.
• Week (1): Two milliliter of solution (B) and (C) was injected to each group of rabbit separately.
• Week (3): Five milliliter of blood was collected by bleeding the rabbits using cardiac puncture.
• Week (4): Booster injection, two ml of solution (D) and (E) were injected to each group of rabbit separately
• Week (5, 7, 9, 11) : Five milliliter of blood was collected by bleeding the rabbits using cardiac puncture.

After blood collection, blood was left at room temperature for 5 minutes, then centrifuged at 800 rpm for 10 minutes, serum was transferred to 1 ml eppendrof tube using micropipette, then kept in deep freezer until use.

2.3.3 Detection of antibodies:

2.3.4Ouchterlony double immunodiffusion:

Ouchterlony double immunodiffusion technique was used for antibodies detection according to method described by (Nilsson, 1978).

2.3.5 Enzyme Linked Immune Sorbent Assay (ELISA):

This test was used for detection of Antibody titer (IgG) which produced against Aflatoxin B1; it was performed using Rabbit IgG Titer ELISA Kit which supplied by General Bioscience Company.

2.4 Determination of the toxic effects of Aflatoxin B1:

The acute toxic effects concentrations of aflatoxin B1 in rabbits ranged from 0.3 mg /kg body weight to 1 mg /kg body weight (FAO, 2000), the concentration of acute toxic effects in our experiment was 0.7 mg/kg body weight.

2.4.1Experimental animals:

Twelve New Zealand White rabbits (650 g – 1500 g) (males and females) were used to determine the effect of aflatoxin B1 , reared at an optimal room temperature ranged between 22 – 25°C . Animals were fed on locally prepared diet which formulated from natural ingredients suitable for growing maintenance.

2.4.2 Experimental Design:

Twelve rabbits were divided into four groups, each group contains (3) rabbits. The groups were as follows:

Group 1: Control animals without any treatments.

Group 2: Animals were intraperitonealy treated with Aflatoxin B1 solution (0.7 mg / kg b.w.).

Group 3: Animals were immunized with 100 µg of aflatoxin B1 – BSA Conjugate using immunization schedule which mentiond as in (2.2.7.3). This group of animals was intraperitonealy treated with Aflatoxin B1 (0.7 mg / kg b.w.).

Group 4: Animals were immunized with 200 µg of aflatoxin B1 – BSA Conjugat. This group of animals was intraperitonealy treated with Aflatoxin B1 (0.7 mg / kg b.w.).

2.5 Immunohistochemical study:

2.5.1Section preparation:

All rabbits were sacrificed after 72 h of the treatment. Liver kidney and spleen were dissected out. Organs were fixed in plastic containers containing 100 ml of formalin 10 %. After that samples were dehydrated in progressively more concentrated alcohols, then embedded in paraffin and cut into section of 4-5 µm thickness (Luna, 1968).

2.5.2 Immunohistochemistry

Immunohistochemical detection Kit of rabbit anti - Cox 2 antibodies supplied from ABCAM Company was used.

2.5.3Staining protocol:

Fixed samples were dewaxed, and the epitopes in the sections were revealed by being heated at 95°C in 10 mmol of citrate acid buffer at pH 6, for 10 min in a microwave, and then were left at room temperature for 20 min. The slides were then washed twice in PBS (pH 7.5) for 5 min. Tissue sections were incubated with primary anti-COX-2 antibodies and diluted 1:100 at room temperature in a humid chamber for 1 h. After being washed with PBS, slides were incubated with the secondary antibody, horseradish peroxidase goat anti-rabbits IgG, for 1 h, in a humid chamber, at 4°C, then washed with PBS and incubated with the 3,3′ diaminobenzidine substrate for 7 min and counter-stained with Harris hematoxylin, clarified in xylene, and mounted.

2.5.4Evaluation of Immunohistochemistry Results:

Immunohistochemical signal specificity was demonstrated by the absence of immunostaining in the negative control slides and its presence in recommended positive controls, clear brown cytoplasmic staining pattern were considered positive, The extent of staining was scored using the following scale: 0 = (negative), 1 = (weak positive), 2 (moderate positive), 3 (strong positive), 4(severe positive) (Davidson *et al*., 2004).

2.6 Statistical Analysis

The mean ± Standard Error (SE) values were calculated for each group to determine the significant of intergroup difference. Differences were considered significant at $P \leq 0.05$.

3. Results

The results of positive COX-2 immunostaining in rabbit´s liver, kidney and spleen was detected as brown cytoplasmic staining, while there is no brown stain in negative results, figures (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12). IHC expression results of COX-2 were illustrated in table (1).

Table 1: COX-2 IHC expression

Organs	Groups			
	G1	G2	G3	G4
Liver	0.00±0.00*	4.00±0.00*	3.00±0.00*	1.00±0.00*
Kidney	0.00±0.00*	3.66±0.33*	1.33±0.33*	0.00±0.00
Spleen	0.00±0.00*	2.00±0.00*	2.33±0.33	0.00±0.00

Each value expressed as Mean ± Standard Error (SE) of three replicates.
G1 = Control (rabbits without treatment).
G2 = rabbits treated with 0.7 mg/ kg aflatoxin B1 (I.P).
G3 = Immunized rabbits (100 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P).
G4 = Immunized rabbits (200 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P). *= Significant ($P \leq 0.05$).

From table mentioned above results showed that there is no expression of COX-2 in the liver of control animals (score = 0), while the expression of COX-2 was significantly high ($P \leq 0.05$) in animals treated with aflatoxin B1 (0.7 mg/kg) only (score = 4) comparing with control animals, the expression of COX-2 in immunized animals (100 µg aflatoxin B1-BSA Conjugate) was significantly lower than the animals treated with aflatoxin B1 only(score = 3), while the expression of COX-2 in immunized animals (200 µg aflatoxin B1-BSA Conjugate) was significantly low ($P \leq 0.05$) (score=1) in compared with immunized animals (100 µg aflatoxin B1-BSA Conjugate).

Results of the expression of COX-2 in kidney showed that there was no expression of COX-2 in control animals (score=0), while the expression of COX-2 in animals treated with aflatoxin B1 (0.7 mg/kg) only was significantly high ($P \leq 0.05$) (score = 3) comparing with control animals, the expression of COX-2 in immunized animals (100 µg aflatoxin B1-BSA Conjugate) was significantly low ($P \leq 0.05$) (score =1) in compared with animals treated with aflatoxin B1 (0.7 mg/kg) only, while there was no expression of COX-2 in immunized animals (200 µg aflatoxin B1-BSA Conjugate) (score=0).

In spleen, results revealed that there was no expression of COX-2 in control animals, while the expression of COX-2 in animals treated with aflatoxin B1 (0.7 mg/kg) only was significantly high ($P \leq 0.05$)(score = 2) in compared with control animals, otherwise There was no significant difference between immunized (100 µg aflatoxin B1-BSA Conjugate) and animals treated with aflatoxin B1 (0.7 mg/kg) only (score = 2), whereas there was no expression of COX-2 in immunized animals (200 µg aflatoxin B1-BSA Conjugate) (score = 0).

Immunohistochemical Finding (COX-2 expression)

1. Liver

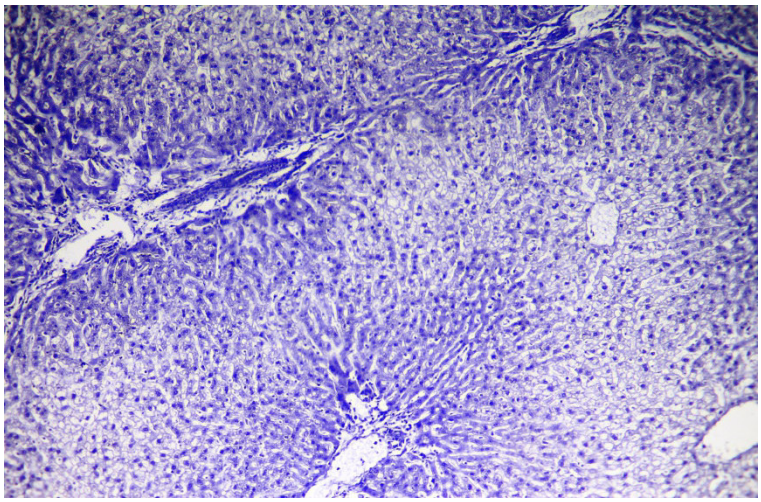

Fig1:(G1) Control animals (score = 0), 10X

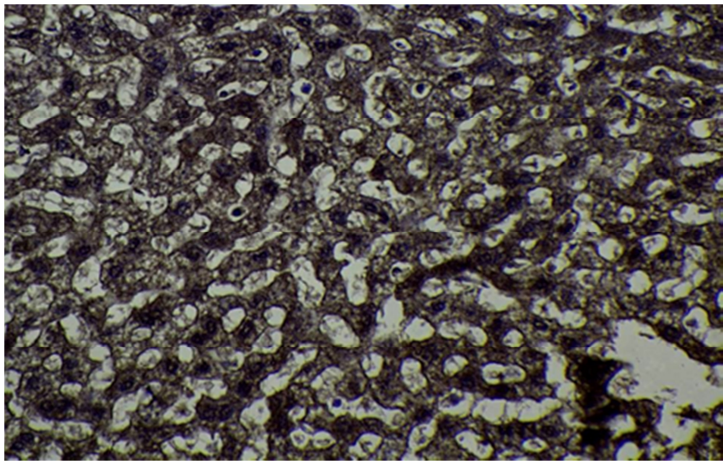

Fig 2 :(G2) Animals treated with aflatoxin B1 (0.7 mg/ kg aflatoxin B1 (I.P)) (score =4), 40X.

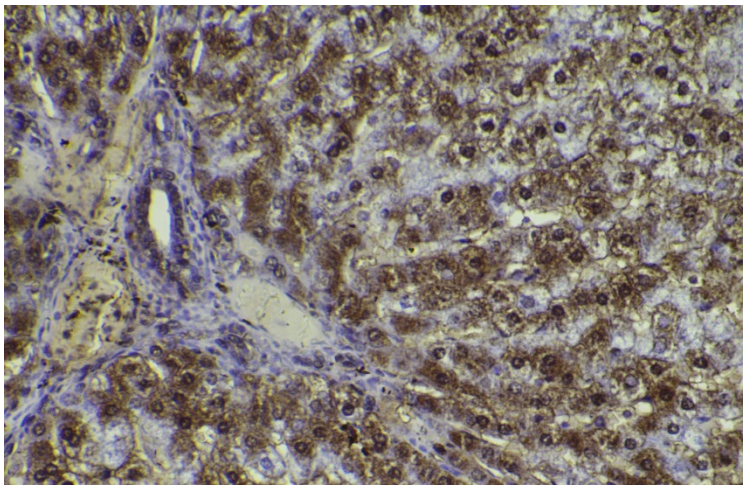

Fig3 :(G3) Immunized animals (100 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=3), 40X.

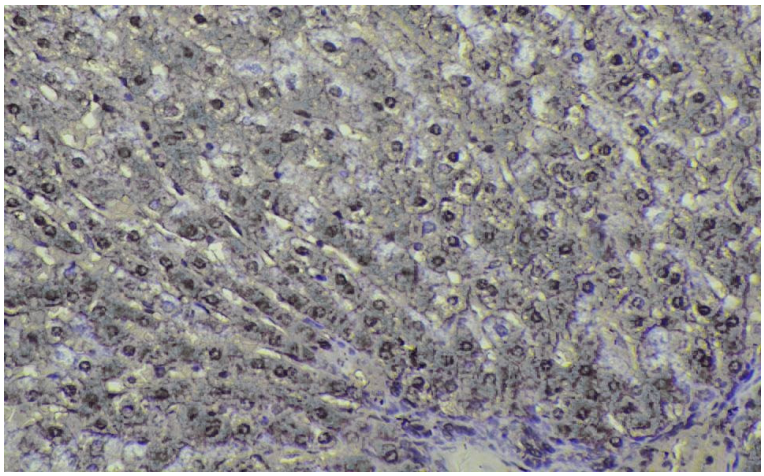

Fig4: (G4) Immunized animals (200 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=1), 40X.

2. Kidney

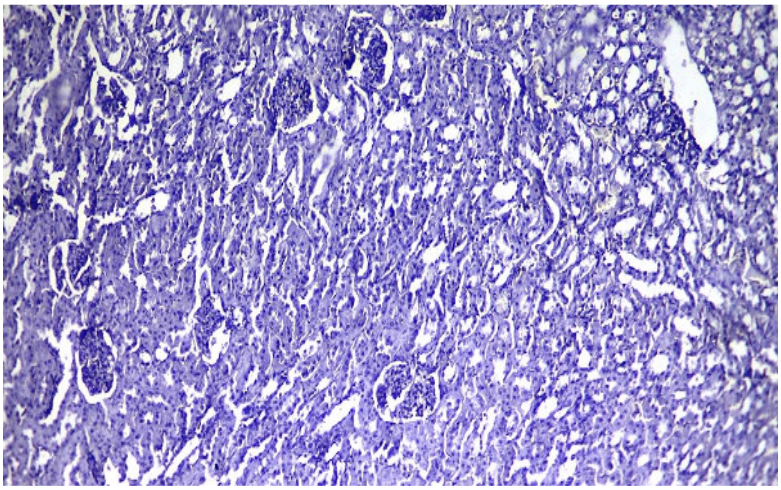

Fig 5 :(G1) Control animals (score = 0), 10X

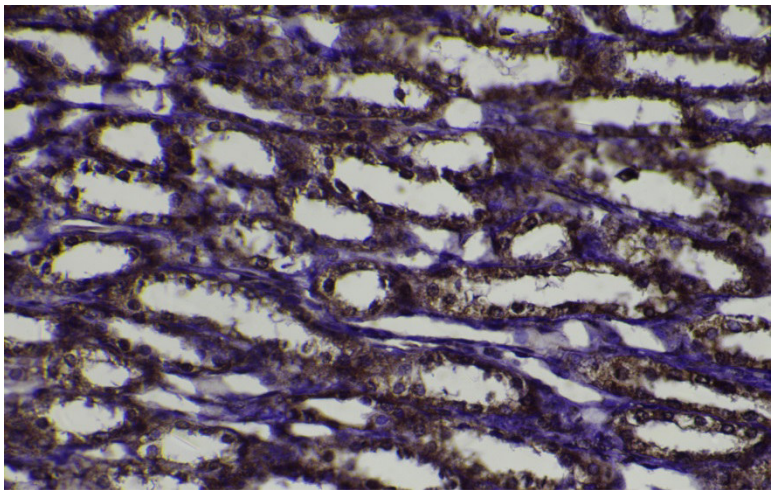

Fig 6 :(G2) Animals treated with aflatoxin B1 (0.7 mg/ kg aflatoxin B1 (I.P)) (score =3), 40X.

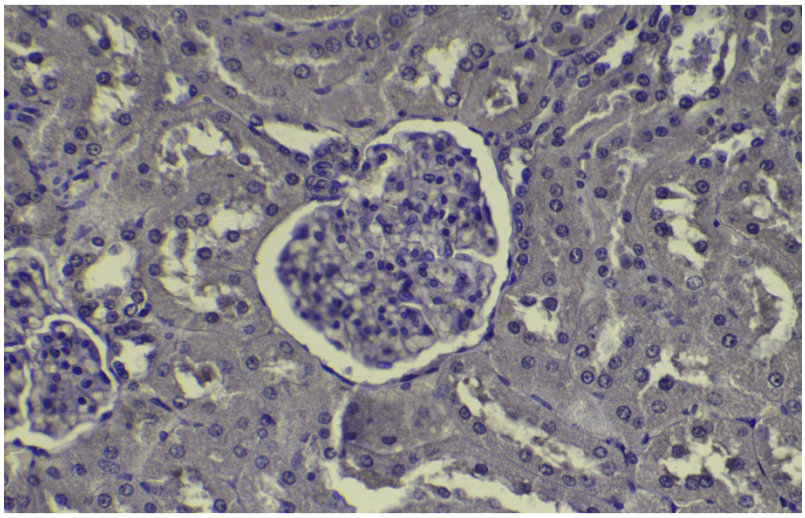

Fig 7:(G3) Immunized animals (100 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=1), 40X.

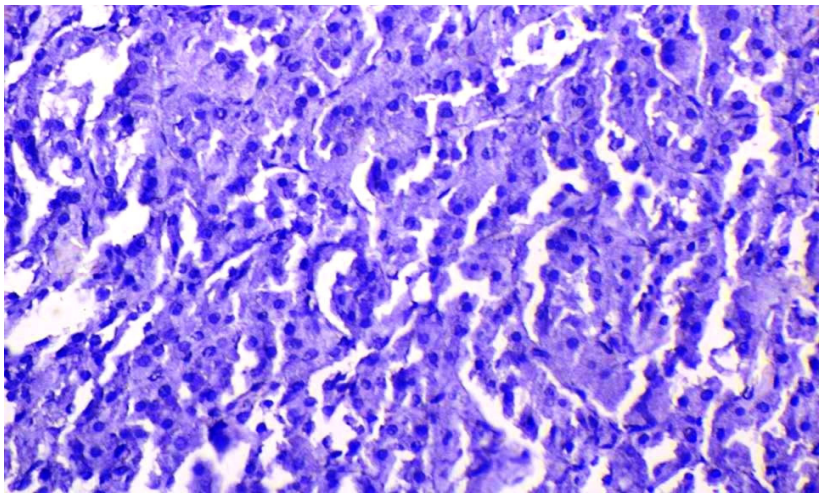

Fig 8: (G4) Immunized animals (200 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=0), 40X.

3. Spleen

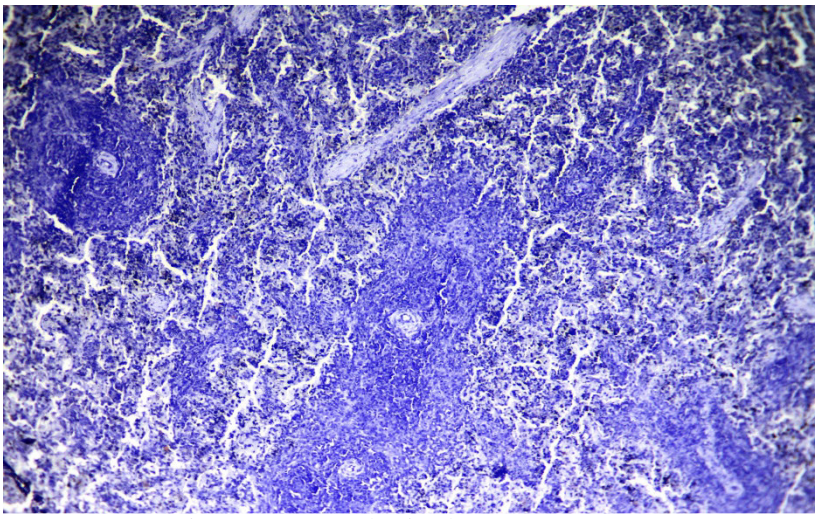

Fig 9:(G1) Control animals (score = 0), 10X

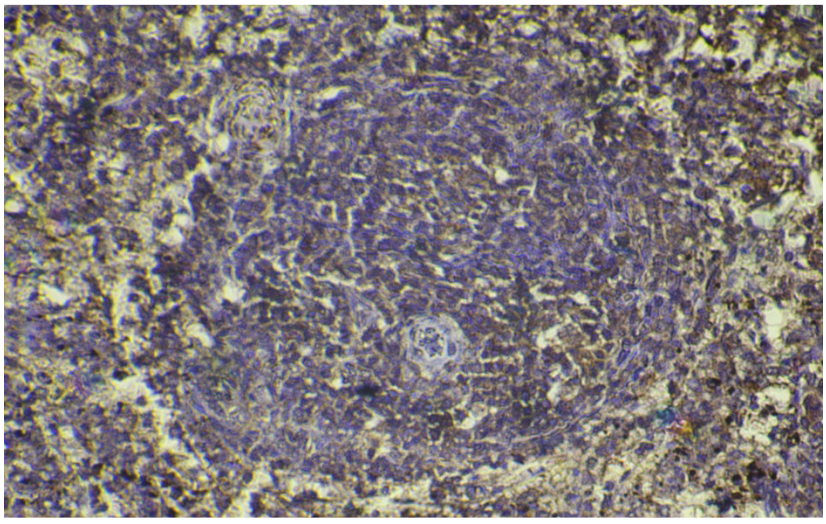

Fig 10 :(G2) Animals treated with aflatoxin B1 (0.7 mg/ kg aflatoxin B1 (I.P)) (score =2), 40X.

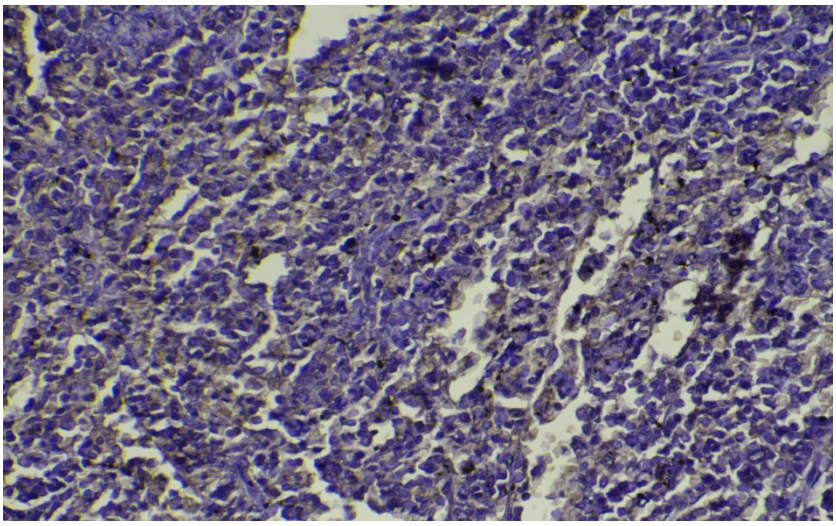

Fig11 :(G3) Immunized animals (100 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=2), 40X.

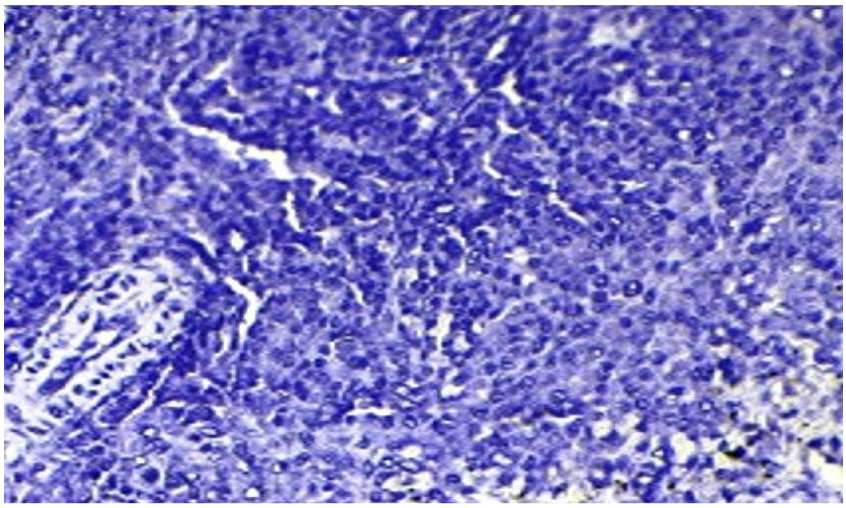

Fig 12: (G4) Immunized animals (200 µg aflatoxin B1-BSA Conjugate) treated with 0.7 mg/ kg aflatoxin B1 (I.P) (score=0), 40X.

4. Discussion

Our results showed that there was no expression of COX-2 in control animals and the expression of COX-2 was high in rabbit's liver, kidney and spleen treated with aflatoxin B1 only due to that Aflatoxin B1 has a range of biological activities, including acute toxicity, teratogenicity, mutagenicity and carcinogenicity (McLean and Dutton, 1995). The International Agency for Research on Cancer (IARC) has classified aflatoxin B1 as the most important known carcinogenic compound (group 1), particularly related to hepatocarcinoma (Henry *et al*., 1999). COX-2 was an enzyme involved in inflammatory processes and Inappropriate up-regulation of COX-2 has been frequently observed in various premalignant and malignant tissues (Mohan and Epstein, 2003), COX-2 has been further implicated in tumorigenesis by increased susceptibility of COX-2 over-expressing transgenic mice (Muller *et al*.,2002) and relative resistance of COX-2 knockout animals to spontaneous or experimentally-induced carcinogenesis (Tiano *et al*., 2002).

COX-2 expression was lower in the liver, kidney and spleen of immunized animals (100 and 200 µg aflatoxin B1-BSA Conjugate) than animals treated with aflatoxin B1 only, because of Lower mortality and reduction of acute toxic effects in rabbits immunized with aflatoxin B1-1(O-carboxymethyl) oxime conjugated to bovine serum albumin (BSA) (Chu and Ueno, 1977; Odunola and Uwaifo, 1998; Odunola and Uwaifo, 2000), on the other hand the expression of COX-2 in immunized animals with100 µg aflatoxin B1-BSA Conjugate was higher than immunized animals with 200 µg aflatoxin B1-BSA Conjugate due to the effectiveness of vaccine in concentration 200 µg in reduce the toxic effect of aflatoxin B1 in rabbits more than 100 µg, these results were agreed with (Ali, 2013) which revealed that immunization of animals against aflatoxin B1 play important role

in reducing of the toxic effects of aflatoxin B1, especially when 200 µg aflatoxin B1-BSA Conjugate was used which shows more effective in reducing this toxic effects on liver, kidney and spleen in comparison with 100 µg aflatoxin B1-BSA Conjugate.

5. Conclusion

In conclusion, the expression of COX-2 was high in liver, kidney and spleen of animals treated with aflatoxin (0.7 mg/kg) only, and the immunization of animals treated with aflatoxin B1 by aflatoxin B1 BSA-conjugate(100 µg and 200 µg) make the expression of COX-2 reduce or remove, on the other hand using 200 µg of vaccine was more effective in reduction or removal the expression of COX-2 than 100 µg.

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