

Acute and Chronic Effects of Aflatoxin on the Liver of Rats During the Storage of Walnuts

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Abstract: This study was conducted on thirty albino male rats, weighting 130 ± 5 g and classified into three groups which were (10 rats each) The control (-ve) fed on standard diet all over the period of the experiment. The first group fed on basal diet containing 2% walnut which was stored for four months. The second group fed on basal diet containing 4% walnut which was stored at the same period and same condition. The study was assigned for eight weeks. The results revealed that, the aflatoxins in the walnut after two months and four months of storage showed a significant increase in AFB₁ and AFG₁ when comparing with control (-ve) group. 4% walnut rat group and 2% walnut group showed non significant difference in food intake, while showed a significant decrease in weight gain and FER compared with control (-ve) group. There were significant decreases in serum HG, PCV, in blood and in serum TP, albumin and globulin, HDL-C, GSP, SOD, also in liver TG, Glycogen, GSH, SOD and GSP of 2% walnut rat group and 4% walnut group in comparing with control (-ve) group. While, there was significant increase in, blood glucose and in serum of bilirubin, ALT, AST, ALP, γ GT enzymes activity, CHO, TG, LDL-C, VLDL-C, T Lipid, P Lipid, MDA. Also, there was significant increase in liver CHO, T Lipid and MDA of 2% walnut rat group and 4% walnut group in compared with control (-ve) group. On the basis of these finding it can be concluded that the highest value of estimated aflatoxins in walnut was appeared after four months of storage and this level show toxic effect on 2% walnut and 4% walnut rats groups which lead to disturbances in some biochemical and histopathological in male rats. Thus, the study recommends eating walnut without storing it for long period.

Key words: Aflatoxins • Antioxidants • Storage • Walnut

INTRODUCTION

Walnut are not only delicious, they also pack a nutritional punch. Walnut has amazing health benefits. Walnut improve cardiovascular function and have wonderful anti-inflammatory properties which improve Sleep as The body's pineal gland produces the hormone melatonin that induces sleep and helps regulate sleep. This hormone is found in walnut-making walnut a great evening or bedtime snack for improving your sleep [1]. Although high in fats and concentrated in calories, the fats in walnut are healthy fats. And walnut is a great source of fiber and protein. As a result, eating walnut helps you feel full and feel satisfied. This is good news for dieters [2]. Walnuts are a great source of anti-inflammatory omega-3 fatty acids, an essential fatty acid

that the body can't manufacture. Its anti-inflammatory, antioxidant defense protects healthy cells from the damaging effects of free radicals. Walnut improves blood vessel health as Omega-3 fatty acids help prevent erratic heart rhythms, make blood less likely to clot and impact how much plaque accumulates in blood vessels. The essential amino acids, l-arginine, helps blood vessels relax and remain smooth, improving the elasticity of the blood vessel. This means improved mental function and protection against heart disease and stroke [3]. Aflatoxins (AF) comprise a family of natural toxins produced by *Aspergillus moulds*. Although at least 20 different forms of AF exist, AF forms B₁, B₂, G₁ and G₂ are the most prevalent and the most toxic forms occurring in plant-based foods. AF is a potent liver carcinogen specially AFB₁. AF infests corn and corn products, nutmeats, dried

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fruits, grains and spices grown and/or stored under hot, humid conditions. Most human exposure to aflatoxins through the diet has been determined to come from the consumption of AF-contaminated nuts and corn [4].

Aflatoxins are toxic and among the most carcinogenic substances known. After entering the body, aflatoxins may be metabolized by the liver to a reactive epoxide intermediate or hydroxylated to become the less harmful aflatoxin [5]. High-level aflatoxin exposure produces an acute hepatic necrosis, resulting later in cirrhosis and/or carcinoma of the liver. Acute hepatic failure is made manifest by hemorrhage, edema, alteration in digestion, changes to the absorption and/or metabolism of nutrients and mental changes and/or coma. They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high-humidity environment or damage from stressful conditions such as drought, a condition that lowers the barrier to entry. Favorable conditions include high moisture content (at least 7%) and high temperature [6].

This study was designed to investigate the levels of aflatoxins stored walnut and the hazardous effects of these levels on some biochemical and histopathological in male rats.

MATERIALS AND METHODS

Materials

Walnut: Walnut was obtained from local market in Kuwait. The walnut was authenticated in the Botany Department, Faculty of Agriculture, Cairo University, Egypt.

Experimental Animals: Thirty of white albino rats (Sprague dawley strain), weighing 130 ± 5 g, provided from National Research Centre, Dokki, Giza, Egypt. The animals were kept under observation for five days before experiment and fed on standard diet and water *adlibitum*. The standard diet comprised of casein (200g/kg), corn starch (497g/kg), sucrose (100g/kg), cellulose (30g/kg), corn oil (50g/kg), mineral mixture (100g/kg), vitamin mixture (20g/kg) and DL-methionine (3g/kg). The standard diet was performed according to Nelson [7].

Storage of Experimental Walnut and Determination of Aflatoxins: Experimental walnut samples were chemically analyses for estimation of aflatoxins at first (zero time) then stored for four months at room temperature.

Aflatoxins were also estimated at two month of storage and at the last of the period of storage (four months). The determination of Aflatoxins B₁, B₂, G₁ and G₂ was conducted using HPLC method [8].

Preparation of Walnut Powder: Walnut was crushed into fine particles as far as possible and stored kept in the refrigerator at 4°C during use [9].

Methods

Experimental Animal Design: The rats were randomly classified into three groups (10 rats each). The control (-ve) fed on standard diet all over the period of the experiment. The first group fed on basal diet containing 2% walnut was stored for four months. The second group fed of basal diet containing 4% walnut stored same period time at same condition. During the study, the food intake was calculated daily and the body weight gain was recorded weekly. Food efficiency ratio (FER) was calculated according to Chapman *et al.* [10]. The experiment continued for 8 weeks. After the experimental period, blood was collected from overnight fasted rats of each group by cardiac puncture. Then the rats were sacrificed for the study of liver biochemical and histopathological parameters. Also, kidneys for each rat were collected for histopathological examination. Part of blood from sacrificed rats was heparinized for estimation blood hemoglobin (HG), packed cell volume (PCV) and glucose according to Drabkin [11], Mc Inory [12] and Trinder [13]. Other part of each blood samples put into a refrigerator for 2 hour then centrifuged for 10 minutes at 3000 rpm to separate serum. Serum was carefully aspirated and transferred into dry clean washer-man tubes by using a Pasteur pipette and kept frozen at (-20°C) till analysis. Serum aspartate and alanine amino transferase (AST&ALT), serum alkaline phosphatase (ALP) and gamma glutamyl transferase enzymes were estimated by using commercially available kits according to Reitman and Frankel [14], Kind and King [15] and Henry [16]. Serum total protein and albumin were measured according to the method described by Weichselbaum [17] and Doumas and Biggs [18]. Serum total bilirubin, creatinine and urea were estimated according to Jendrassik [19], Bonsens and Taussky [20] and Patton and Crouch [21]. Serum cholesterol (CHO), triglycerides (TG), high density lipoprotein cholesterol (HDL-C) and total lipids were determined by using enzymatic colorimetric methods according to Allain *et al.* [22], Buccolo and David [23], Kostener [24] and Schmitc [25].

Blood glutathione peroxidase (GSP) and superoxide dismutase (SOD) enzymes were estimated according to the methods of Misra and Fridovich [26] and Winterbourn *et al.* [27]. The method of Draper and Hadley [28] was employed in determining malondialdehyde (MDA). Liver cholesterol (CHO), total lipids, triglyceride and glycogen were determined according to Richmond [29], Folch *et al.* [30], Scheletter and Nussel [31] and Rerup and Lundquist [32]. The level of liver glutathione S-transferase (GST), superoxide dismutase (SOD) and glutathione peroxidase (GSP) activity was determined by the method of Misra and Fridovich [26], Habig *et al.* [33] and Weiss *et al.* [34]. The extent of lipid peroxidation was estimated in liver homogenates by measurement of malondialdehyde formation using the thiobarbituric acid method [35]. Other parts of liver and kidney collected and immersed in 10% neutral buffered formalin as fixative and sent to Cancer Institute for histopathological examination according to Bancroft *et al.* [36].

Statistical Analysis: Differences between groups were analyzed using Dunnet's *t*-test followed by analysis of variance (ANOVA) [37].

RESULTS AND DISCUSSION

The statistical data in Table 1 presented that, the percentage of aflatoxins at zero time of storage showed a significant increase in AFB₁ and AFG₁ ($p < 0.001$). Data in Tables 2 and 3, indicated that aflatoxins in the walnut after two months and four months of storage showed significant increase in AFB₁ and AFG₁. The statistical data in Table 4 presented that, 4% walnut rat group and 2% walnut group showed non significant difference in initial weight and food intake, while showed a significant decrease in weight gain ($p < 0.01$) and FER ($p < 0.001$) compared with control (-ve) group. The data in Table 5 showed that significant ($p \leq 0.001$) ($p \leq 0.01$) decrease in

serum hemoglobin, packed cell volume of 2% walnut rat group and 4% walnut group, there was significant increase in, glucose 2% walnut rat group and 4% walnut group in compared with control (-ve) group. These results were in agreement with those obtained by Blomhoff *et al.* [38], Reddy *et al.* [39], Huff *et al.* [40] and Mohiuddin *et al.* [41], which reported that aflatoxin causes hematopietic suppression and anemia observed as decreases in total erythrocytes, packed-cell volume and hemoglobin. The activity of serum or plasma enzymes has been extensively used as a measure of aflatoxins activity in rats. Increased activities of sorbitol dehydrogenase, glutamic dehydrogenase lactate dehydrogenase alkaline phosphatase, acid phosphatase, aspartate aminotransferase and alanine aminotransferase were reported in aflatoxicated chickens [42-45]. The increase in the levels of serum enzymes measured was interpreted as a consequence of hepatocyte degeneration and subsequent leakage of enzymes [46].

Data presented in Table 6 showed that 4% walnut rat group followed 2% walnut group showed a significant increase in serum ALT, AST, alkaline phosphatase and Gamma glutamyl transferase enzymes activity ($P < 0.001$) compared with control (-ve) rat group. These results were in agreement with those obtained by Bankole and Mabekoje [47], who reported that the enzymatic activity depends upon the extent of inflammatory or necrobiotic damage to the hepatocytes and residual parenchymal mass. Data in Table 7 showed that, 4% walnuts rat group and 2% walnuts group showed a significant increase in serum bilirubin ($P < 0.001$) and significant decrease in serum total protein, albumin and globulin ($P < 0.05$ & 0.01) in compared with control (-ve) group. The Aflatoxin decreases total serum proteins, alpha, beta and gamma globulins [48]. Total serum proteins contents are depressed due to reduced values of alpha and beta globulins and albumin, while gamma globulins are affected more variably [49].

Table 1: Mean values \pm SD of AFB₁, AFB₂, AFG₁ and AFG₂ aflatoxins in the Walnut at first (zero time) of the storage.

Aflatoxins				
Samples	AFB ₁ ($\mu\text{g/kg}$)	AFB ₂ ($\mu\text{g/kg}$)	AFG ₁ ($\mu\text{g/kg}$)	AFG ₂ ($\mu\text{g/kg}$)
Walnut	1969.09 \pm 211.61 a	0.46 \pm 0.03c	176.74 \pm 25.11 a	16.08 \pm 2.71b

Values with the same letters indicate non- significant difference ($P < 0.05$) and vice versa.

Table 2: Mean values \pm SD of AFB₁, AFB₂, AFG₁ and AFG₂ aflatoxins in the walnut at two months of storage.

Aflatoxins				
Samples	AFB ₁ ($\mu\text{g/kg}$)	AFB ₂ ($\mu\text{g/kg}$)	AFG ₁ ($\mu\text{g/kg}$)	AFG ₂ ($\mu\text{g/kg}$)
Walnut	3854.18 \pm 298.71a	2.11 \pm 0.79d	3521.11 \pm 201.31a	48.37 \pm 8.71c

Values with the same letters indicate non- significant difference ($P < 0.05$) and vice versa.

Table 3: Mean values \pm SD of AFB₁, AFB₂, AFG₁ and AFG₂ aflatoxins in the Walnut at four months of storage.

Samples	Aflatoxins			
	AFB ₁ (μ g/kg)	AFB ₂ (μ g/kg)	AFG ₁ (μ g/kg)	AFG ₂ (μ g/kg)
Walnut	4922.7 \pm 477.34a	1.31 \pm 0.35d	4401.88 \pm 392.61a	40.31 \pm 5.77b

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

Table (4): Mean values \pm SD of body weight gain, food intake and FER of control, 2% and 4% walnut and consumed experimental rat groups.

Groups	Variables			
	Initial weight (g)	Weight gain (g)	Food intake (g)	FER
Control (-ve)	130.57 \pm 5.50a	150.20 \pm 13.21a	16.31 \pm 1.71a	0.130 \pm 0.003a
2%Walnut	131.67 \pm 5.60a	110.65 \pm 11.33b**	16.35 \pm 1.61a	0.098 \pm 0.006c***
4%Walnut	132.96 \pm 4.81a	114.81 \pm 12.13b**	18.71 \pm 1.36a	0.089 \pm 0.005c***

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

FER: Food efficiency ratio.

Table 5: The Mean values \pm SD of blood HG, PCV and glucose of control, 2% and 4% walnut consumed experimental rat groups.

Groups	Variables		
	HG (g/dl)	PCV%	Glucose (mg/dl)
Control (-ve)	13.78 \pm 1.79a	37.24 \pm 4.41a	95.71 \pm 5.29c
2%Walnut	9.65 \pm 1.15c**	25.99 \pm 5.31bc**	141.32 \pm 13.61a***
4%Walnut	8.91 \pm 1.01bc***	21.71 \pm 2.17bc***	139.41 \pm 14.51a**

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

HG: Hemoglobin. PCV: Packed cell volume.

Table (6) :The Mean values \pm SD of serum ALT, AST, ALP and γ GT of control, 2% and 4% walnut consumed experimental rat groups.

Groups	Variables			
	AST(μ /ml)	ALT(μ /ml)	ALP (μ /ml)	γ GT (μ /ml)
Control(-ve)	49.67 \pm 5.31b	37.33 \pm 3.45c	47.10 \pm 4.95c	5.021 \pm 0.85c
2%Walnut	65.96 \pm 6.91a**	56.75 \pm 6.09a***	68.16 \pm 7.08a***	9.11 \pm 1.31a***
4%Walnut	87.23 \pm 8.91 a***	80.41 \pm 9.27a***	95.31 \pm 9.11a***	10.41 \pm 1.30a***

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

AST: Aspartate aminotransferase. ALT: Alanine aminotransferase. ALP: Alkaline phosphatase

γ GT: Gamma glutamyle transferase.

Table 7: The Mean values \pm SD of serum bilirubin, TP, albumin and globulin of control, 2% and 4% walnut consumed experimental rat groups.

Groups	Variables			
	Bilirubin (mg/dl)	TP (g/dl)	Albumin (g/dl)	Globulin (g/dl)
Control (-ve)	0.55 \pm 0.03b	7.69 \pm 1.10a	3.71 \pm 0.42a	3.99 \pm 0.77a
2%Walnut	1.73 \pm 0.30a***	5.41 \pm 0.78b*	3.31 \pm 0.20a	2.17 \pm 0.29b*
4%Walnut	1.96 \pm 0.65a***	5.10 \pm 0.55b*	2.99 \pm 0.33ab	2.11 \pm 0.33b**

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

TP: Total protein.

Table 8: Mean values \pm SD of serum on lipid profile CHO, HDLc, LDLc, VLDLc, TG, T Lipids and P Lipids of control, 2% and 4% walnut consumed experimental rat groups.

Variables							
Groups	CHO (mg/dl)	HDLc (mg/dl)	LDLc (mg/dl)	VLDLc (mg/dl)	TG (mg/dl)	T Lipids (mg/dl)	P Lipids (mg/dl)
Control(-ve)	93.17 \pm 8.67c	31.41 \pm 4.41a	48.74 \pm 5.66c	13.02 \pm 1.78b	65.14 \pm 7.25b	422.71 \pm 99.67b	264.4 \pm 57.71b
2%Walnut	169.61 \pm 15.51a***	24.40 \pm 3.17b**	128.06 \pm 17.19a***	17.14 \pm 1.36a**	85.71 \pm 9.65a**	669.99 \pm 114.18a***	414.68 \pm 76.71a***
4%Walnut	173.98 \pm 21.21a***	17.81 \pm 1.81 b***	137.41 \pm 14.17a***	18.74 \pm 2.18a**	93.71 \pm 9.80a***	788.71 \pm 151.31a***	521.04 \pm 105.61a***

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

CHO: Cholesterol HDLc: High density lipoprotein cholesterol

LDLc: Low density lipoprotein cholesterol TG: Triglyceride VLDLc: Very low density lipoprotein cholesterol

T Lipid: Total lipids P Lipid: Phospholipids

Table 9: The Mean values \pm SD of blood GSP, SOD and MDA of control, 2% and 4% walnut consumed experimental rat groups.

Variables			
Groups	GSP (mmol/l)	SOD (mmol/l)	MDA (mmol/l)
Control (-ve)	8.35 \pm 1.37a	25.26 \pm 7.01a	3.22 \pm 0.25c
2%Walnut	5.71 \pm 0.97b**	18.32 \pm 2.11b***	6.78 \pm 0.82ab***
4%Walnut	4.88 \pm 0.66b***	13.61 \pm 1.98bc***	8.55 \pm 1.31a***

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

GSP: Glutathione-peroxidase. SOD: Superoxide dismutase. MDA: Malondialdehyde.

Table 10: The Mean values \pm SD of Liver cholesterol, T lipids, TG and glycogen of control, 2% and 4% walnut consumed experimental rat groups

Variables				
Groups	CHO (mg/dl)	T Lipids (mg/dl)	TG (mg/g)	Glycogen (mg/100g)
Control (-ve)	4.17 \pm 0.65c	38.67 \pm 6.81b	3.11 \pm 0.61a	5.11 \pm 1.31a
2%Walnut	6.51 \pm 1.18a**	51.93 \pm 5.24a*	2.11 \pm 0.22b*	3.12 \pm 0.77b**
4%Walnut	7.24 \pm 1.11a***	55.21 \pm 5.51a***	1.67 \pm 0.21b**	2.47 \pm 0.35b**

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

CHO: Cholesterol. T lipids: Total lipids TG: Triglycerides.

Table 11: The Mean values \pm SD of Liver GSH, SOD, GSP and MDA of control, 2% and 4% walnut consumed experimental rat groups.

Variables				
Groups	GSH (μ /mg)	SOD (μ /mg)	GSP (μ /mg)	MDA (mmol/g)
Control (-ve)	12.18 \pm 2.18a	45.71 \pm 6.11a	55.11 \pm 6.24a	20.77 \pm 2.33c
2%Walnut	8.31 \pm 1.32b**	21.73 \pm 2.12c***	36.70 \pm 4.61bc***	28.60 \pm 4.12a***
4%Walnut	6.71 \pm 1.21b***	18.71 \pm 1.51c***	27.78 \pm 3.11b**	34.21 \pm 4.31a***

Significant with control (-ve) group * P<0.05 ** P<0.01 *** P<0.001

Values with the same letters indicate non- significant difference (P<0.05) and vice versa.

GSH: Glutathione SOD: Superoxide dismutase. GSP: Glutathione-peroxidase MDA: Malondialdehyde.

The statistical data in Table 8 showed that, 4% walnut rat group and 2% walnut group showed that, 4% walnut rat group caused significant increase in cholesterol, triglycerides, LDL-C, VLDL-C, total lipids and phospholipids, while there was significant decrease in HDL-C in compared with control (-ve) group. From data in Table 9 it could be observed that significant ($p \leq 0.001$) ($p \leq 0.01$) decrease in serum glutathione-peroxidase, superoxide dismutase of 4% walnut rat group and 2% walnut group, there was significant increase in, malondialdehyde 2% walnut rat group and 4% walnut group in compared with control (-ve) group. Data in

Table 10 showed that 4% and 2% walnut rat groups caused significant ($p \leq 0.001$) ($p \leq 0.01$) increase in cholesterol and total lipid. While there was significant ($P < 0.05$) ($P < 0.01$) decrease in liver triglyceride and glycogen compared with control (-ve) group. These results were in agreement with those obtained by Singh [50], who indicated that increase in liver lipids was confined mainly to phospholipids and cholesterol, whereas triglycerides showed a significant decrease. Effect of walnut on Liver GSH, SOD, GSP and MDA of control, 2% walnut and 4% walnut rat groups in Table 11 showed that 4% followed 2% walnut rat group caused

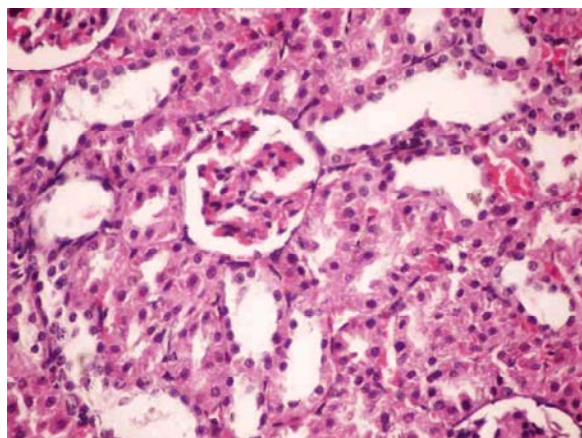


Photo 1: Kidney of rat from control negative group showing the normal histopathological structure of renal parenchyma (H and E \times 200).

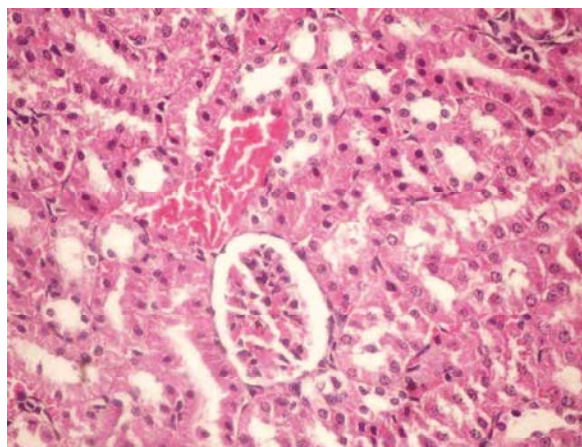


Photo 2: Kidney of rat from group administered 2% Walnuts showing interstitial nephritis (H and E \times 200).

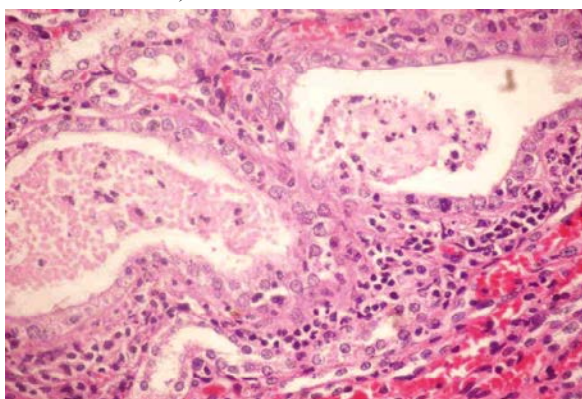


Photo 3: Kidney of rat from group administered 4% Walnuts revealed cystic dilatation of renal tubules with cellular cast in their lumen (H and E \times 200).

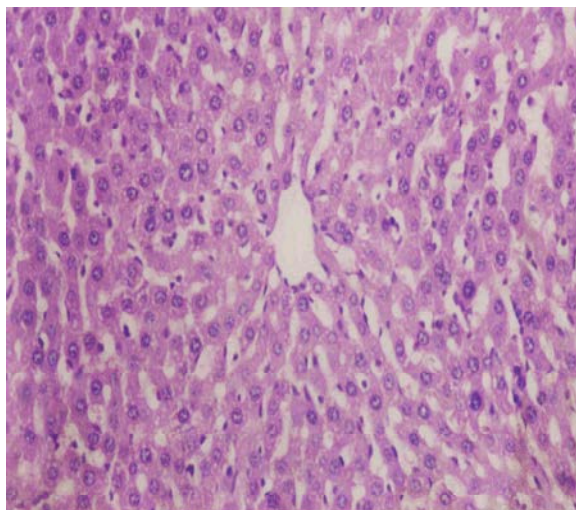


Photo 4: Liver of control (-ve) rat group showing the normal histological structure (H and E \times 200).

significant ($p \leq 0.001$) ($p \leq 0.01$) decrease in glutathione, superoxide dismutase and glutathione-peroxidase while, there was significant ($P < 0.05$) ($P < 0.01$) increase in liver Malondialdehyde compared with control (-ve) group. These results were in agreement with those obtained by El-Agamy [51], who show that AFB(1) significantly decreased the level of GSH and the activities of superoxide dismutase and GPX and increased level of malondialdehyde aflatoxin B1 toxicity in rats.

The obtained results are confirmed by the histopathological examination, kidney of control negative rat revealed the normal histopathological structure of renal parenchyma (Photo 1). Conversely, kidney of rat from group administered 2% Walnut revealed interstitial nephritis (Photo 2). Examined kidney sections from group which administered 4% Walnut revealed cystic dilation of renal tubules with cellular cast in their lumen (Photo 3). Moreover, the obtained results of live are confirmed by the histopathological examination. Liver of control (-ve) group showed normal histological structure of hepatic lobules, which consists of central vein and concentrically, arranged hepatocyte (Photo 4). Liver of rat from group administered 2% Walnut showed liver of hypercholestrimic rat showing kupffer cells activation and perivascular mononuclear cells infiltration (Photo 5). While, liver of rat from group administered 4% Walnuts showed vacuolar degeneration of hepatocytes, congestion of hepatic sinusoids and hepatic necrosis with inflammatory cell infiltration (Photo 6).

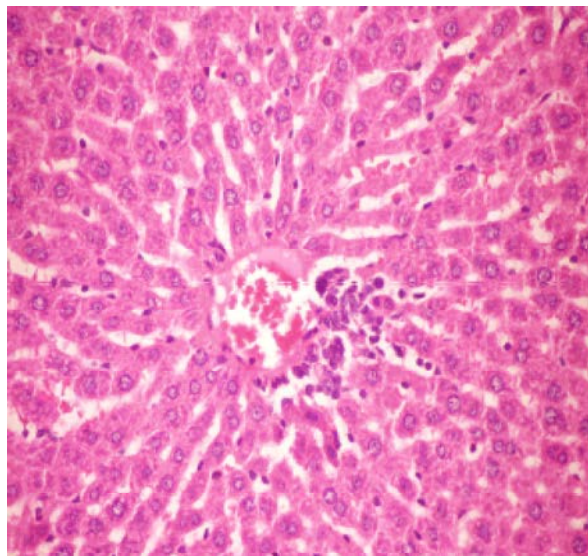


Photo 5: liver of hypercholesterimic rat showing kupffer cells activation and perivascular mononuclear cells infiltration (H and E X400)

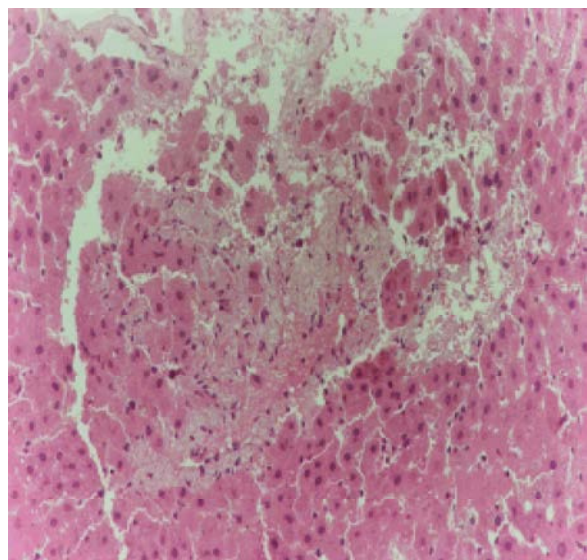


Photo 6: Liver of control (+ve) rat group showing vacuolar degeneration of hepatocytes, congestion of hepatic sinusoids and hepatic necrosis with inflammatory cell infiltration (H and E X 200)

CONCLUSIONS

On the basis of these finding it can be concluded that the highest value of estimated aflatoxins in walnut was appeared after four months of storage and this level show toxic effect on 2% walnut and 4% walnut rats groups which lead to disturbances in some biochemical and

histopathological in male rats. Thus, the study recommends eating walnuts before four months of storage.

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