Monitoring the Preventive Effect of Hydrogen Peroxide and γ-Radiation of Aflatoxicosis in Growing Rabbits and the Effect of Cooking on Aflatoxin Residues

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The objective of the present study was the prevention of aflatoxicosis of growing rabbits fed aflatoxin (AF)-contaminated diet (833 μg of aflatoxins/kg) using 5% hydrogen peroxide (H_2O_2) and γ-radiation at a dose level of 500 krad (5 kGy) and fed to growing rabbits. A total of 24 New Zealand white rabbits were divided into three groups. The experimental diets included AF-contaminated diet; AF-decontaminated diet, and AF-free diet (control). The obtained data showed significant reduction ($p < 0.05$) in live body weight and body weight gain of rabbits that fed on AF-contaminated diet as well as AF-decontaminated diet relative to control. There were no differences in feed consumption among the three groups; feed efficiency reduced significantly for AF-contaminated and AF-decontaminated groups. Mortality percentage was 25% for AF-contaminated and AF-decontaminated groups. Relative weight of the liver increased in animals fed AF-contaminated and AF-decontaminated diets, whereas the relative weight of kidneys decreased for both. There was no difference in total protein, but the levels of albumin and globulin were altered in rabbits receiving AF-contaminated diet. Serum enzymes (alanine amino transferase and aspartate amino transferase) activity increased significantly in rabbits that received AF-contaminated as well as AF-decontaminated diets. Histopathological examination revealed particularly alteration in liver and kidneys of rabbits fed AF-decontaminated diet. Results showed that the percentage of aflatoxin reduction ranged between 67 and 80% in boiled liver and between 79 and 90.5% in fried liver, whereas complete reduction in AF was found after boiling followed by frying. These findings indicate that the use of H_2O_2 and γ-radiation for the destruction of aflatoxins in contaminated diet induces adverse effects in the animals.

Keywords: *Aflatoxicosis; aflatoxins; hydrogen peroxide; γ-radiation; cooking; rabbit*

INTRODUCTION

Aflatoxins (AFs) are potent hepatotoxins and carcinogenic and are naturally occurring toxins produced minly by the molds *Aspergillus flavus* and *Aspergillus parasiticus* (*1*, *2*) that contaminate a variety of agricultural foods and feed products. Because contamination of food and feeds with mycotoxins can occur despite the most strenuous effects at prevention and because contamination can occur in the field before harvest and/or during storage (*3*), practical and economical detoxification procedures for the feeds have developed with the understanding that such techniques should be utilized if preventive measures have failed and not as alternatives to good culture and storage practices (*4*).

Any detoxification process must be technically and economically feasible if it is applied practically. The FAO requirements for an acceptable decontamination process stipulate that the procedure must (1) destroy, inactivate, or remove AFs; (2) not produce or leave toxic and/or carcinogenic/mutagenic residues in the final products or in food products obtained from animals fed on decontaminated feeds; (3) not significantly alter the important technologic properties and, ideally, (4) destroy fungal spores and mycelium that could proliferate and produce new toxins under favorable conditions. Several approaches to detoxification have been utilized, including physical separation of contaminated kernels or seeds, biological degradation, and chemical reaction with acids, bases, organic solvents, and gases (*5*−*8*). An aflatoxin detoxification process using H_2O_2 (0.3 or 0.5%) completely prevented fungal growth and AF production for up to 90 days (*9*). Treatment of dried fig fruits containing 25 ppb of aflatoxin B_1 (AFB_1) and with H_2O_2 (0.2%) results in 65.5% AF reduction in 72 h (*10*). Maximum inactivation (98%) of AFM_1 was obtained using 1% H_2O_2 plus 0.5 nM riboflavin added to contaminated milk (*11*). Reduction of AFB_1 in corn and soybean occurred between 10 and 20 kGy of irradiation (*12*).

Therefore, the purpose of this research was twofold: (1) toxicological studies and histopathological figures in rabbit and (2) the effect of different treatments on AF residues in certain edible tissue (i.e., liver) after different types of cooking (boiling and/or frying).

MATERIALS AND METHODS

Materials. The aflatoxins were produced through the fermentation of corn by *A. parasiticus* NRRL 2999 (*13*). The fermented corn was autoclaved and ground to a powder, and the corn powder was incorporated into the basal diet to provide the desired level of 833 μg/kg of diet.

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Detoxification Procedure. The contaminated diet was treated with 5% hydrogen peroxide (H_2O_2) and irradiated at 500 krad (5 kGy) of γ-radiation (*14*).

Kits. Transaminase [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], total protein (TP), albumin, and globulin kits were obtained from Bio Merieux, France.

Animals. One-month-old male New Zealand white rabbits (1 kg body weight) were selected from the Nile Co., Cairo, Egypt, and maintained on standard rabbit diet (protein, 16.3%; fat, 2.5%; crude fibers, 14%; and metabolic energy, 2670 kcal/kg) and water ad libitum.

Experimental Design. After an acclimation period of 1 week, 24 rabbits were divided randomly into three groups (8 rabbits/ group), including the control group, the AF-contaminated (833 μg/kg of diet; 135.6, 14.6, 636, and 46.8 μg of AFB_1, AFB_2, AFG_1, and AFG_2/kg, respectively) group, and the AF-decontaminated group. The animals were maintained on these treatments for 6 weeks with a feed consumption ration of 6% ration/rabbit/day. After the end of the experimental period, the animals were slaughtered and the internal organs of each rabbit (liver, lungs, heart, and kidney) were removed, weighed, and subjected to clinical examination. Blood samples were collected from all rabbits within different treatment groups from ear vein in dry glass tubes and left to clot. Blood serum was separated by centrifugation at 3000 rpm for 15 min. Serum was analyzed colorimetrically for total protein, albumin, globulin, and transaminase (AST and ALT).

Determination of AF Residue. The aflatoxins (B_1, B_2, G_1, and G_2) in the ration were extracted according to an AOAC method (*15*). Meanwhile, AF residues in liver and kidney were extracted and cleaned up according to the method of Stubblefied (*16*). The quantitative determination of AFs was carried out using HPLC (Waters) supplied by model 420 fluorescence detector (excitation, 338 nm; and emission, 455 nm) and C_{18} Nova-Pak column (15 × 0.44 cm). Mobile phases included solvent A [a mixture of acetonitrile plus water (23+77, v/v)] and solvent B methanol (*15*). The average recovery of aflatoxins was 84−87%.

Histopathological Examination. The liver and kidney were excised and fixed in 10% buffered neutral formalin. Samples from each tissue were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin; sections of 6 μm were cut and stained with hematoxyline and eosin (H, E) as described by Culling (*17*) and then examined under a light microscope.

Cooking Methods of Rabbit Liver. *Boiling* was done by placing a weight of liver in a covered cooker that contained 3 times that weight of water with or without 2% sodium bisulfite and then heated to boiling for 15 min. *Frying* was done in hot butter at 140 °C for 5 min.

Statistical Analysis. Data of all parameters were statistically analyzed as a completely randomized design by using analysis of variance according to SAS (*18*); significant differences among treatment means were statistically tested by using Duncan's new multiple-range test (*19*).

RESULTS AND DISCUSSION

The biological effects of aflatoxins resulting from ingestion of contaminated diet by animals emphasized the potential public health hazard that might arise from AF-contaminated food. Most lesions caused by AFs in animals occurred mostly in growth retardation, reducing fed intake and liver cells and blood serum.

The effect of H_2O_2 (5%) and radiation (5 kGy) on AFs is presented in Table 1. Results indicated that AFs were completely detoxified by 5% H_2O_2 and radiation (5 kGy). Other investigators have positive results in aflatoxins with γ-radiation and H_2O_2 (*20*−*22*); however, Patel (*14*) reported that 5% H_2O_2 and 4 kGy of γ-radiation were required for total degradation of 100 μg of AFs.

Table 1. Concentration of Aflatoxins (Micrograms per Kilogram) in the Diets

	AFs (μg/kg)				
type of diet	B_1	B_2	G_1	G_2	total
control corn	ND[a]	ND	ND	ND	ND
AF-contaminated	135.6	14.6	636	46.8	833
AF-decontaminated	ND	ND	ND	ND	ND

[a] ND, not detectable.

Table 2. Effect of Feeding of AF-Contaminated and AF-Decontaminated Diets on Body Weight Gain, Feed Consumption, Feed Efficiency, and Mortality Percent in Rabbits[a]

	diet		
parameter	control	aflatoxins	AFs + H_2O_2 + radiation
initial body wt (kg)	1.128	1.135	1.128
final body wt (kg)	1.878a ± 0.09	1.592b ± 0.18	1.599b ± 0.17
wt gain (kg)	0.750a ± 0.02	0.457b ± 0.11	0.471b ± 0.11
feed consumption/ animal (kg)	4.734a ± 1.1	4.199a ± 0.58	4.510a ± 0.73
feed efficiency (gain/ kg of feed)	0.160a ± 0.07	0.110b ± 0.04	0.100b ± 0.05
dressing wt (kg)	1.132a ± 0.6	0.872b ± 0.5	1.000a ± 0.01
mortality (%)	0	25	25

[a] In each column means having the same letter are not significantly different.

Table 3. Effect of Feeding of AF-Contaminated and AF-Decontaminated Diets on Relative Organ Weight (Percent) in Rabbits[a]

	treatment		
organ	control	AFs	AFs + H_2O_2 + radiation
liver	5.13a ± 0.15	5.57b ± 0.25 (+7.9%)	5.37b ± 0.22 (+4.7%)
lung	1.01a ± 0.16	1.05a ± 0.09 (+4%)	1.20b ± 0.12 (+18.8%)
kidney	1.16a ± 0.12	1.0b ± 0.15 (−13.8%)	0.97b ± 0.13 (−16.4%)
heart	0.45a ± 0.11	0.47a ± 0.10 (+4.4%)	0.53b ± 0.10 (+17.8%)

[a] In each column means having the same letter are not significantly different.

The effects of AF-contaminated and AF-decontaminated diets on rabbit performance are presented in Table 2. It is clearly indicated that body weight was decreased significantly between AF-contaminated or AF-decontaminated groups and the control group. However, there is no significant difference between AF-contaminated and AF-decontaminated groups. All groups showed normal signs of feed consumption ranging from 4.199 to 4.734 kg/head. No significant differences were detected between control and AF-contaminated as well as AF-decontaminated groups. On the other hand, feed efficiency ratio was significantly decreased in rabbits fed AF-contaminated diet and AF-decontaminated diet compared to control group. This may be the causative of the diminished weight gain of those groups (*23*, *24*). Data revealed that the treatment of AF-contaminated diet with 5% H_2O_2 and γ-radiation did not improve growth performance of rabbits. This reduction of live body weight (LBW) of rabbits receiving AF-contaminated diet could be due to the disturbance of protein synthesis by aflatoxins (*23*−*26*).

Clinical examination of rabbits fed AF-contaminated diet revealed an injury in the intestinal tract and hemorrhages. These observations may be due to chemical interaction occurred between the toxin and H_2O_2 by several mechanisms, including alterations in absorption, protein binding, and excretion of one or both interacting

Table 4. Effect of Feeding on AF-Contaminated or AF-Decontaminated Diet on Serum Biochemical Values[a]

treatment	total protein (g/100 mL)	albumin (g/100 mL)	globulin (g/100 mL)	ALT (μmol/L)	AST (μmol/L)
control	6.83a ± 0.11	4.11bc ± 0.13	2.73bc ± 0.11	27.20b ± 1.20	121.4b ± 4.40
AFs	6.36a ± 0.13	2.76c ± 0.13	3.60a ± 0.31	38.60a ± 1.80	175.0a ± 6.2
AF + H_2O_2 + radiation	6.56a ± 0.11	3.14bc ± 0.11	3.42ab ± 0.11	37.90a ± 5.60	176.3a ± 4.2

[a] In each row means having the same letters are not significantly different.

toxicants (*25*); this interaction perhaps caused growth retardation of rabbits in this group (*24*). Decrease of dressing weight of rabbits receiving AF-contaminated or AF-decontaminated diets referred to the decrease in weight gain (Table 2). Mortality percent (25%) was observed in groups of rabbits receiving AF-contaminated and AF-decontaminated diets. Diazy and Sugahara (*24*) observed the same percent of mortality in chicks receiving AF-contaminated diet (4 mg of AF/kg of diet).

Relative weights of liver, heart, lungs, and kidney are presented in Table 3. These results indicated that rabbits fed AF-contaminated diet had an increase in the relative weights of liver; lungs, and heart (7.9, 4, and 4.4%, respectively) and a decrease in the relative weight of kidney (−13.8%). Moreover, AF-decontaminated diet results in increased relative weights of liver, lungs, and heart (4.7, 18.8, and 17.8%, respectively), whereas the relative kidney weight decreased (−16.4%). These results regarding liver weight supported the earlier findings that AF caused hypertrophy of the liver and were in agreement with the observations of other investigators (*23*, *24*, *27*) who reported that adding AF to the diet significantly increased the relative liver weights. However, the decrease in the relative kidney weights may be related to the retardation of protein synthesis by AF (*28*).

The biochemical effects of feeding on AF-contaminated and AF-decontaminated diets and control groups on biochemical parameters regarding liver functions are presented in Table 4. The data showed an insignificant decrease in serum total protein (6.36 g/100 mL) and a significant decrease in serum albumin (2.76 g/100 mL) but an increase in serum globulin (3.60 g/100 mL) in the AF-contaminated group. The AF-decontaminated group showed the same trend in serum total protein (6.56 g/100 mL), serum albumin (3.14 g/100 mL), and serum globulin (3.42 g/100 mL). These findings indicated that both AF-contaminated and AF-decontaminated treatments resulted in a stress on liver function and supported the results of other studies that found a reduction in the level of albumin and an increase of globulin, indicating disturbance in protein synthesis (*23*, *25*, *29*, *30*).

The activities of serum AST (175 and 176.3 μmol/L) and ALT (38.6 and 37.9 μmol/L) in AF-contaminated and AF-decontaminated groups, respectively, showed increases (Table 4). Serum ALT and AST activities are considered sensitive indicators of liver function. It should be mentioned that serum ALT and AST activities are associated with cell necrosis of different tissues (*31*). It is understood that AF induces tissue necrosis and cell damage of rabbits fed AF-contaminated diet that increases activity of ALT and AST enzymes (*23*, *25*, *32*, *33*). On the other hand, the increase of AST and ALT activities of the group fed AF-decontaminated diet may be due to the presence of H_2O_2 in the diet, which causes such chemical interaction with aflatoxins presented in the diet as recognized above resulting in the disturbance of liver function as well as enzyme activity (*24*, *34*).

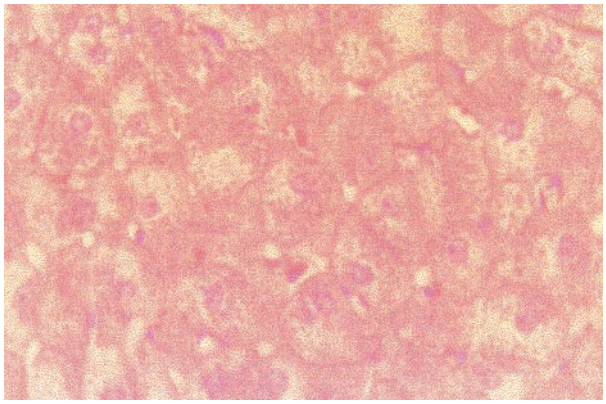

Figure 1. Coagulative necrosis of hepatic cells with complete lysis of their nuclei.

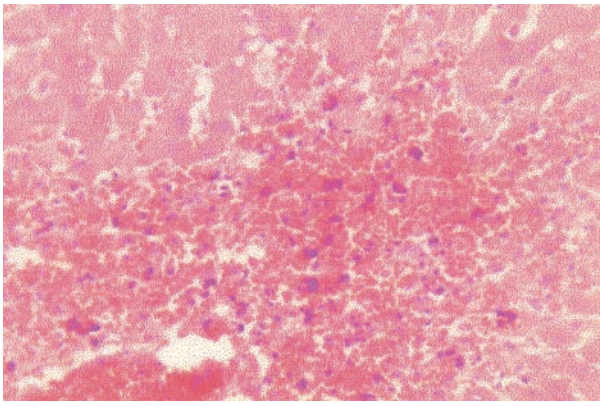

Figure 2. Liver area of hemorrhages with necrosis of hepatic parenchyma and presence of hemosiderine pigments engulfed by Kupffer cells.

Histopathological studies of liver specimens of group fed AF-contaminated diet revealed dilation of central veins and blood sinusoids, hepatic cells swollen and granular, proliferation and hyperplasia of the bile duct epithelium forming the large bile duct. The proportion of binucleated cells had increased. In some cases the examination revealed scattered areas of hemorrhage with necrosis of the hepatic parenchyma and the presence of hemosiderin pigments either free or engulfed by Kupffer cells (Figure 1). Examination of kidney specimens of the same group revealed congestion with areas of hemorrhages. The renal tubular cells were swollen and granular. Some renal tubules contained homogeneous eosinophilichyaline casts (Figure 3).

Some rabbits in the AF-decontaminated group showed swollen and vacuolated hepatic cells (Figure 2), whereas kidneys revealed congestion with small areas hemorrhage (Figure 4). These findings are similat to those of other investigators (*25*, *34*).

With regard to the determination of AFs in liver and kidney tissues of different groups, Table 5 indicates that all test samples from liver and kidney were free from AFs; however, the livers of the AF-contaminated group revealed the presence of AFB_1 with wide variation.

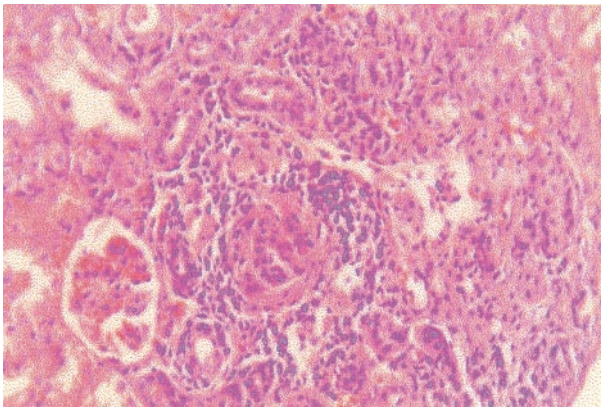

Figure 3. Kidney atrophy and necrosis of the renal parenchyma and replaced by round cells and fibroblasts, glomeruli in the area replaced by fibrous tissue.

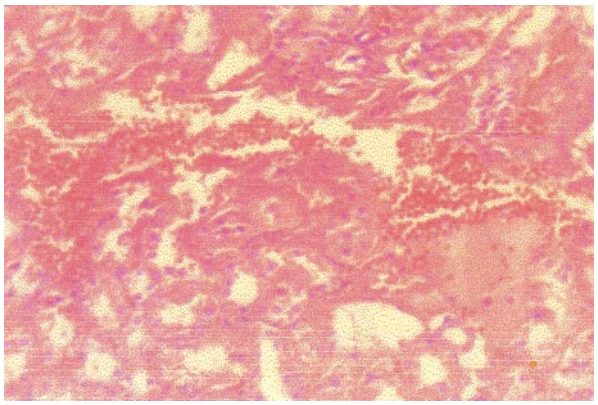

Figure 4. Kidney area of hemorrhage between the renal tubles.

Table 5. Determination of Aflatoxin Residues in Liver of Rabbit Fed AF-Contaminated or AF-Decontaminated Diet

animal	AFs (μg/kg)	AFs + H_2O_2 + radiation	control
1	27.8	ND[a]	ND
2	33.4	ND	ND
3	29.5	ND	ND
4	35.5	ND	ND
5	24.4	ND	ND
6	30.0	ND	ND
7	28.3	ND	ND
8	34.1	ND	ND

[a] ND, not detectable.

Previous studies on feeding of contaminated diet with mycotoxin led to detectable concentration in some organs. AFB_1 was detected in hen eggs, liver and kidney of cattle, rabbits, hens, swine, pig, and fish (*35*, *36*).

Results in Table 6) indicate that AF levels could be reduced by the different means of cooking. The destruction of AFB_1 during cooking of liver in boiling water (100 °C for 15 min) varied from 67.8 to 80% with an average of 74.6%, whereas boiling water with 2% $NaSO_4$ recorded more reduction (87−95%) with an average of 91.3%. Similarly the destruction of AFB_1 varied from 79 to 90.5% with average of 83.7% when liver was fried in hot butter. On the other hand, boiling followed with frying in hot butter resulted in a complete reduction of AFB_1 in the livers of animals fed AF-contaminated feed. These results are consonant with the observation (*28*) that the destruction of AFs increased with the increased moisture level. Boiling parts for 30 and 60 min was found to produce 68 and 80% reduction, respectively (*37*). Moreover, autoclaving and frying yielded a corn snack with almost complete reduction of aflatoxins (*38*), and cooking contaminated maize with oil reduced AFB_1 by 94%. These variations could be attributed to treatment, cooking process, matrix, length of treatment, presence of protein, and pH (*39*).

Table 6. Effect of Cooking on Aflatoxin Residues in Liver Tissues of Rabbits Fed Contaminated Diet

concn of AF in liver								
initial	boiling (100 °C/15 min)		boiling with 2% $NaHSO_4$		boiling and frying		frying (140 °C/5min)	
μg/kg	μg/kg	%	μg/kg	%	μg/kg	%	μg/kg	%
27.80	5.56	80	1.39	95	ND[a]	100	3.34	88
33.40	8.18	75.5	4.34	87	ND	100	7.01	79
29.50	6.49	78	2.95	90	ND	100	5.02	83
35.30	11.36	67.8	3.93	88.9	ND	100	6.94	80.3
24.40	4.88	80	1.22	95	ND	100	2.44	90
30.00	9.00	70	2.70	91	ND	100	4.81	84
28.30	7.20	74.6	1.98	93	ND	100	2.68	90.5
34.10	8.94	73.8	2.70	92.1	ND	100	6.81	80
30.35	7.7	74.6	2.65	91.3	ND	100	4.95	83.7

[a] ND, not detectable.

CONCLUSION

Decontamination of aflatoxins by H_2O_2 and radiation was an efficient procedure to eliminate the AFs from contaminated diet in a short time, although it failed to produce a safe final decontaminated product when biologically evaulated using growing rabbits. This might be due to the formation of another toxic compound when using H_2O_2 and radiation that affects the performance of the tested rabbit. On the other hand, boiling with frying in hot butter (140 °C/5 min) of rabbit liver from the AF-contaminated group was more effective in AF reduction of contaminated tissues, compared with other tested methods.

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