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ORIGINAL ARTICLES

The Effect of Aflatoxin M_1 Exposure on Oxidant/Antioxidant Status of the Flour Millers with Emphasis on 8-Hydroxy-2-Deoxyguanosine

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ABSTRACT

Aflatoxins (AFs) are produced by toxigenic fungi after undergoing biosynthesis pathway involving several enzymes and reactions. Exposure to aflatoxin leads to several health-related conditions including acute and chronic aflatoxicosis, aflatoxin-related immune suppression, liver cirrhosis and cancer. This study aims to emphasize the effects of AFs M_1 on the oxidant/antioxidant status and estimates 8-hydroxy-2-deoxyguanosine (8-OHdG) as biomarker of oxidative DNA damage in flour millers. It included 95 millers divided according to their job description into 4 categories; storage, garbling, grinding and packaging workers. From each individual, blood samples were collected for the determination of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT). Also urine samples were collected for the estimation of Aflatoxin M_1, 8-OHdG and creatinine (cr). The results showed that the level of AFM_1/Cr was significantly higher in grinding workers compared to workers in the other three departments. 8-OHdG/Cr and CAT were significantly higher in the grinding workers than in the packaging workers. There were significant correlations between AFM_1/Cr and 8-OHdG/Cr in the storage and grinding workers, and between AFM_1/Cr and MDA in grinding workers. While, there were negative correlations between AFM_1/Cr and SOD in the packaging workers and between AFM_1/Cr, CAT and GPx in the grinding and packaging workers. SOD and CAT were negatively correlated with MDA in the storage workers. Also, GPx was negatively correlated with the MDA in the grinding workers and with 8-OHdG/Cr in the packaging workers. **Conclusions** the results showed a decline in the activities of the antioxidant enzymes in erythrocytes and plasma; specially among grinding workers which may be due to their exposure to high concentrations of aflatoxin. Further studies are needed to assess the antioxidant status in flour millers.

Key words: Aflatoxin M_1 (AFM_1), 8-hydroxy-2-deoxyguanosine (8-OHdG), Superoxide dismutase, Glutathione peroxidase, Catalase, Malondialdehyde.

Introduction

Aflatoxins (AFs) are secondary metabolites of some mold strains of *Aspergillus; mainly A. flavus* and *A. parasiticus* that frequently contaminate cereal crops during production, harvest, storage or processing (Eaton and Gallagher, 1994; Nigam *et al*., 1994; Krishnan *et al*., 2009). *Aspergillus* produces four major aflatoxins B_1, B_2, G_1, and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2), that are known to be carcinogenic to both humans and animals, of which AFB_1 is the most potent hepatotoxic and hepatocarcinogenic agent (Ardic *et al*., 2008; Hamid *et al*., 2013). Susceptibility to AFs toxicity is significantly different between persons; according to their differing abilities to detoxify aflatoxin by biochemical processes, and their age and gender (Williams *et al*., 2004).

Aflatoxin M_1 (AFM_1) is the hydroxylated metabolite of AFB_1 formed in liver by means of cytochrome P450-associated enzymes (Cavaliere *et al*. 2006). It causes toxic and carcinogenic effects (Galvano *et al*. 1996; Cavaliere *et al*. 2006). AFB_1 is also metabolized by cytochrome-P450 enzymes to the reactive intermediate AFB_1-8, 9 epoxid (AFBO) which binds to liver cell DNA, resulting in DNA adducts. DNA adducts interact with the guanine bases of the liver cell DNA and cause a mutational effect in the P53 tumor suppressor gene (Bedard & Massey, 2006; Hamid *et al*., 2013).

Aflatoxins especially AFB_1, produce reactive oxygen species (ROS) such as superoxide radical anion, hydrogen peroxide, lipid Hydro peroxides and hydroxyl radical. This hydroxyl radical interacts with DNA and produces mutations (Halliwell; Gutteridge 2006). To control the level of reactive oxygen species and protect cells under stress conditions, living tissues contain enzyme systems, which are capable of reducing the DNA damage, such as, superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) as well as antioxidant substances (Valko *et al*., 2006). SOD protects cells from oxidative damage by breaking down a

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potentially hazardous free radical superoxide ($O_2\cdot$) to H_2O_2 and O_2. The produced H_2O_2 can then be decomposed by CAT and GPx (Kunwar & Priyadarsini 2011).

The effect of reactive oxygen species is balanced by the antioxidant action of non-enzymatic antioxidants, as well as by antioxidant enzymes. Uncontrolled generation of ROS can lead to their accumulation causing oxidative stress in the cells (Mates, 2000; Kohen & Nyska, 2002; Kunwar & Priyadarsini, 2011). Recent studies showed that ROS also have a role in cell signaling, including apoptosis, gene expression and the activation of cell signaling cascades (Hancock *et al*., 2001; Circu and Aw, 2010).

Aim of the Study:

The present study aims to emphasize the effects of Afl M_1 on the oxidant/antioxidant status and estimates 8-hydroxy-2-deoxyguanosine (8-OHdG) as biomarker of oxidative DNA damage in flour millers.

Subjects and Methods:

Wheat Milling Procedure:

Milling is the process by which wheat is ground into flour. This is done through different steps. At first the bales of wheat are carried and evacuated in special places and then the wheat is cleaned from impurities (Garbling workers). Conditioning of the clean wheat in uniform moisture content is an important step to improve separation of bran from the floury endosperm. After conditioning, different batches of wheat are blended to make a mix capable of producing the required flour quality. Then sequence of breaking, grinding and separating operations are done for separation of the bran and germ from the endosperm (Grinding workers). Millers can create variations in features of the flour by blending together the many different flour streams produced by the mill. The last step is packing of the different milled products to be ready for marketing (Packaging workers), or wheat, its impurities, and the packed flour are stored in special places (Storage workers).

Subjects:

The present study included all the flour millers (102 millers) who were working in one of the biggest flour mills in Helwan. The included workers were 95 millers, after exclusion of the millers with positive virus hepatitis B or C. The workers were divided according to their job description into 4 categories; storage workers, garblers, grinders and packers.

Ethical consent was taken from the Ethical Committee of NRC, and written consent was taken from all the included subjects.

Questionnaire:

A questionnaire contained detailed personal, medical, occupational and environmental histories in brief.

Clinical Examination:

General and abdominal examination was done for all the included persons.

Biochemical Analysis:

Urine and blood samples were collected in sterile tubes. Urine samples were used to determine the level of urinary excretion of 8-hydroxy-2-deoxyguanosine (8-OHdG); as a biomarker of oxidative DNA damage, and urinary aflatoxin metabolite AFM_1.

Blood samples were divided into two portions; one left to clot for 30 minute at 37°C and then centrifuged at 3,000 rpm for 10 minutes to isolate the sera for measuring the MDA and CAT activities. The other portion was collected on EDTA for preparing the packed RBCs for the determination of SOD and GPx activities. The sera, the packed RBCs, and the urine were kept at -20°C. SOD activity expressed in U/ml and was measured through spectrophotometer by Biodiagnostic kits, using the method of Nishikimi *et al*. (1972). GPx activity (mU/ml), was measured through spectrophotometer by Biodiagnostic kits, Paglia and Valentine (1967) method. CAT activity was measured through spectrophotometer by Biodiagnostic kits, method of Aebi (1984) and expressed in U/L. MDA was measured by Biodiagnostic kits, method of Ohkawa *et al.* (1979). The concentrations were expressed in nmol/ml. Colorimetric kinetic determination of creatinine (Cr) in urine was performed by using bio-diagnostic kits (www.bio-diagnostic.com)

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Estimation of AFM_1 in Urine:

AFM_1 standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The extraction was carried out according to the method described by Polychronaki *et al*. (2008) and Saad-Hussein *et al*. (2012). Aflatoxins were determined using an HPLC system consisting of a Waters Binary pump Model 1525, a Rheodyne manual injector, a 2475 multi-wavelength fluorescence detector (Ex 365, Em 440 nm) and a data workstation with soft ware Empower 2. A Phenomenax Gemini-NX, C18 (5U, 250-46 mm i.d.) column was used. The mobile phase was water / isopropanol / acetonitrile (80:12:8 v/v) and the separation was performed at ambient temperature at a flow rate of 1.0 mL / min

Each analysis was performed twice, and the results presented were the mean values of the two measurements. Aflatoxin M_1 standards was injected every six injections to assure the correct identification of the urine sample peaks based on their retention times.

Determination of 8-hydroxy-2-deoxyguanosine (8-OHdG) in Urine:

Urinary 8-OHdG analysis was performed according to the method described by Kim *et al*., (2004) and Hussein *et al*. (2012). 8-OHdG was extracted from 1 ml urine. The eluents were evaporated under ultra – pure N_2 stream and reconstituted in 5 ml deionized water for injection in HPLC.

HPLC Condition:

HPLC column for 8-OHdG was C18 (250 × 4.6, particle size 5 µ). The mobile phase consisted of acetonitrite / methanol / phosphate buffer (25/10/965). Phosphate buffer was prepared by dissolving 8.8 g of potassium dihydrogen Phosphate in1000 ml deionized water and pH was adjusted at 3.5. The buffer then filtered 2 times before used. The separation was performed at ambient temperature at a flow rate of 1 ml/min and using electrochemical detector with cell potential 600 mv, the injection volume was 20 µl. The concentration of 8-OHdG was determined by external standard method using peak areas. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. The concentration in samples was obtained from the curve.

Creatinine normalization was done for levels of AFM_1 and 8-OHdG (i.e. dividing of these levels on the levels of creatinine for every subject to obtain the ratio between AFM_1 and 8-OHdG to creatinine (AFM_1/Cr and 8-OHdG/ Cr).

Statistical Analysis:

The collected data were statistically analyzed through SPSS package version 14. Qualitative data were represented as number and percent, and quantitative data as mean ± standard deviation. The qualitative results were analyzed using Pearson's Chi-square (χ^2) and likelihood ratio in case more than 25% of the cells had expected count < 5. Quantitative comparisons were done through analysis of variance test (ANOVA) and the post hoc test, the least significant difference (LSD). The non-parametric measures (Kruskal-Wallis and Mann-whitney) tests were used for quantitative data with skweness . The relationships between the different variables were studied through correlation coefficient. The difference was considered significant at P-value ≤0.05 levels.

Results:

The age of the included millers was in the range 24 - 58 years (47±7.6 years), and their duration of exposure was 5 - 40 years (21.9±8.6 years). There was no significant difference between the workers in different departments relative to their ages, duration of exposure, smoking habits and smoking index. The AFM_1 in urine samples was in average 0.33±0.08 ng/ml, with range 0.0 - 2.28 ng/ml. The average levels of AFM_1 / creatinine (AFM_1/Cr) of the workers was 0.12±0.03 ng/mg, with range 0.0 - 0.98 ng/mg.

Table (1) shows that the level of AFM_1/Cr was significantly higher in grinding workers compared to workers in the other three departments. DNA oxidative damage biomarker (8-OHdG) and catalase (CAT) were significantly higher in the grinding workers than in the packaging workers.

Figure (1) shows two examples; one of urine sample without the peak of 8-OHdG, and the other of a urine sample with 8-OHdG peak.

Table (2) shows that there were significant positive correlations between the levels of AFM_1/Cr and the 8-OHdG/Cr in the storage and grinding workers, and between AFM_1/Cr and MDA in grinding workers. While, there were negative correlations between the levels of AFM_1/Cr and SOD in the packaging workers, and between the levels of AFM_1/Cr and the levels of CAT and GPx in the grinding and packaging workers.

Table 1: Comparison of the levels of AFM_1/Cr, 8-OHdG/Cr, MDA and the antioxidant enzymes between workers in different departments.

	Storage workers (19)	Garbling workers (41)	Grinding workers (13)	Packaging workers (22)	ANOVA	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	F-ratio	P-value
AFM_1/Cr (ng/mg)	$0.077±0.02^{(Gr)}$	$0.098±0.04^{(Gr)}$	$0.313±0.09^{(S,Gar,P)}$	$0.064±0.04^{(Gr)}$	3.255	P= 0.03
SOD (U/ml)	211.3±89.90	248.8±47.47	253.6±60.81	249.8±88.79	1.521	P= 0.22
CAT (U/L)	646.5±110.4	639.8±123.9	$699.5±120.9^{(P)}$	$581.4±107.2^{(Gr)}$	2.931	P= 0.04
GPx (mU/ml)	153.6±20.79	152.7±54.80	104.2±12.34	122.6±21.59	0.154	P= 0.93
	Median (Range)	Median (Range)	Median (Range)	Median (Range)	Kruskal Wallis	
8-OHdG/Cr (ng/mg)	$3.7^{(--)}$ (0.01-69.9)	$4.5^{(--)}$ (0.01-66.9)	$8.2^{(P)}$ (0.01-69.9)	$0.01^{(Gr)}$ (0.01-38.6)	7.259*	P=0.06
MDA (nmol/ml)	5.5 (1.9-39.9)	8.5 (0-25.0)	9.1 (3.2-30.7)	7.1 (1.2-27.9)	3.918*	P= 0.27

S: Storage workers, Gr: Grinding workers, Gar: Garbling workers, P: Packaging workers
ANOVA and LSD were used, *Kruskal Wallis and Mann-Whitney Tests
Mean ± SD were used for ANOVA test, while Median & range were used for Kruskal Wallis test

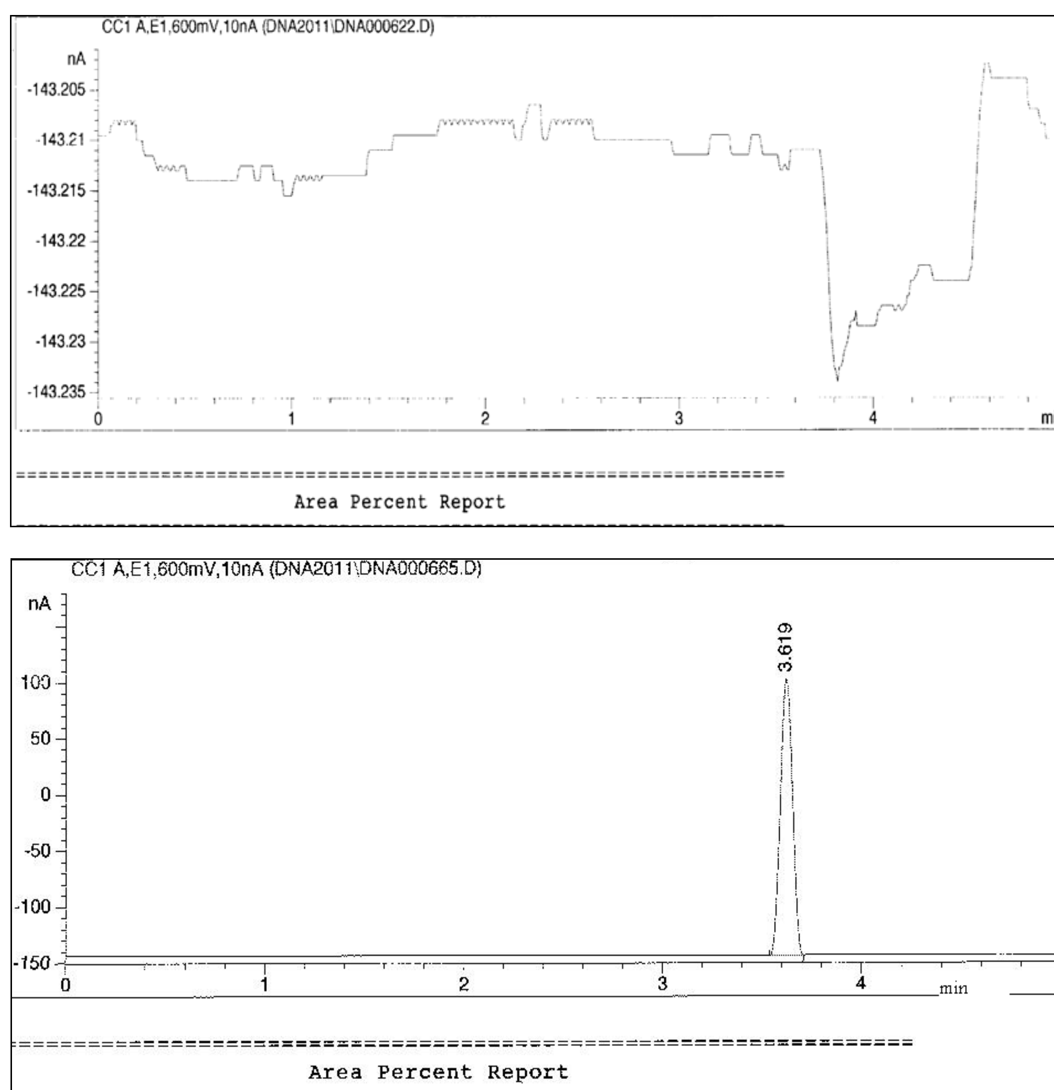

Fig. 1: Peaks of 8-OHdG; the upper chart without the peak of 8-OHdG and the lower chart is with 8-OHdG peak.

Table 2: Relationships between the levels of AFM_1/Cr and the levels of 8-OHdG/Cr, MDA and the antioxidant enzymes in the different departments.

AFM_1/Cr (ng/mg)		Storage workers (19)	Garbling workers (41)	Grinding workers (13)	Packaging workers (22)
8-OHdG/Cr (ng/mg)	r=	0.5	0.3	0.8	0.1
	P-value	0.04	NS	0.01	NS
MDA (nmol/ml)	r=	0.1	0.4	0.5	0.2
	P-value	NS	NS	0.05	NS
SOD (U/ml)	r=	-0.02	0.1	-0.3	-0.5
	P-value	NS	NS	NS	0.04
CAT (U/L)	r=	-0.2	-0.3	-0.5	-0.5
	P-value	NS	NS	0.04	0.03
GPx (mU/ml)	r=	-0.04	-0.2	-0.5	-0.6
	P-value	NS	NS	0.04	0.03

Table (3) shows that in the storage workers, SOD and CAT were negatively correlated with MDA. Also, GPx was negatively correlated with the MDA in the grinding workers and with 8-OHdG/Cr in the packaging workers.

Table 3: Correlations between levels of 8-OHdG/Cr and the levels of MDA and antioxidant enzymes in the different departments.

		Storage workers (19)		Garbling workers (41)		Grinding workers (13)		Packaging workers (22)	
		8-OHdG/Cr (ng/mg)	MDA (nmol/ ml)	8-OHdG/ Cr (ng/mg)	MDA (nmol/ ml)	8-OHdG/ Cr (ng/mg)	MDA (nmol/ ml)	8-OHdG/ Cr (ng/mg)	MDA (nmol/ml)
MDA (nmol/ml)	r=	0.04		0.01		0.8		0.4	
	P-value	NS		NS		0.008		NS	
SOD (U/ml)	r=	-0.1	-0.5	0.02	-0.2	-0.01	-0.02	-0.1	0.3
	P-value	NS	0.04	NS	NS	NS	NS	NS	NS
CAT (U/L)	r=	0.2	-0.6	0.05	-0.1	0.1	-0.03	0.02	-0.2
	P-value	NS	0.01	NS	NS	NS	NS	NS	NS
GPx (mU/ml)	r=	-0.2	-0.1	-0.1	-0.2	-0.02	-0.6	-0.5	-0.1
	P-value	NS	NS	NS	NS	NS	0.04	0.05	NS

Discussion:

Now, it is well known that AFs (B_1, B_2, G_1, and G_2) are food-borne secondary toxic metabolites produced during the growth of *Aspergillus flavus* and *Aspergillus parasiticus* group of fungi. In flour milling process mycotoxins may be redistributed and concentrated in the environment of the workplace, as there is no step or operation that destroys mycotoxins (Ardic *et al*., 2008). Formation of AFs depends on the foods on which the moulds grow and the conditions of heat and humidity during the growth, harvesting and storage of the crops (Creepy 2002). Exposure of mill workers to AFs may be through inhalation or skin contact and sometimes by ingestion if they eat with unwashed hands within workplace (Peraica *et al*., 1999).

In the present study, AFM_1/Cr was found to be significantly higher in grinding workers compared to that in the workers from the other three departments. According to Saad-Hussein project (2010 – 2013), grinding workers were exposed to *Aspergillus flavus* in the respirable dust as the storage workers (76.9 CFU /m^3), lower concentrations compared to the garbling workers (179.4 CFU /m^3), and higher than packaging workers (21.4 CFU /m^3). The significant increase in the AFM_1/Cr levels in grinding workers compared to the workers from the other three departments could be explained by their exposure to higher concentrations of AFs, as mycotoxins tend to be concentrated in germ and bran fractions in the dry milling process as proved by Bulleman and Bianchini (2007). Some previous studies were interested in exposure of Egyptian population to aflatoxin. The study of Piekkola *et al*. (2012) was concerned with maternal exposure to dietary AFB1 during pregnancy. AFM1 was detected in 44 of 93 samples, with a mean concentration 19.7 pg/mg creatinine, while in the study of Polychronaki *et al*. (2008) on healthy Egyptian children aged 1–2.5 years compared to Guinean children aged 2–4 years, overall aflatoxins were less frequently present in Egyptian than Guinean urine samples. For AFM1, the mean level in Guinea (16.3 pg/ml) was 6-fold higher than in Egypt (2.7 pg/ml). They concluded that the frequency and level of these biomarkers in Egyptian population were modest compared with high-risk African countries.

Urinary 8-OHdG an oxidative damage nucleoside, has been widely used as a marker for evaluating in vivo oxidative stress (Valavanidis *et al*., 2009). The levels of 8-OHdG in the current study were higher in the grinding workers compared to the other workers, but were significantly higher only when compared with the packaging workers.

The present results showed a positive correlation between AFM_1/Cr concentrations and the levels of 8-OHdG in storage and grinding workers. Highly reactive substances may combine with DNA bases; such as guanine, to produce alterations in DNA. This may be the most important product from the carcinogenic point of view. Formation of these adducts disrupts the normal working process of the cell, and can ultimately lead to a loss of control over cellular growth and division.

In the same concern, Peng *et al*. (2007) found a significant correlation between AFB_1 exposure and oxidative stress by assessing urinary excretion of 8-OHdG besides other biomarkers. Moreover, AFB_1 treated cells in experimental animals showed increased levels of 8-OHdG as a biomarker of oxidative stress (Shen *et al*., 1995; Guindon *et al*., 2007; Shi *et al*., 2012).

The present study revealed also that the levels of MDA were correlated positively with AFM_1/Cr in the grinding workers. These results are in agreement with the findings reported previously in experimental animals. They showed that lipid peroxidation was significantly increased in the liver. Antioxidant treatments decreased these hazardous effects in AFs treated mice as compared to controls (Yu *et al*., 1988; Verma and Nair, 1999; Uysal and Agar, 2005).

In the present study, there were no significant differences in the levels of antioxidant enzymes between the workers according to their work description, except in CAT. CAT activity was significantly higher in grinding workers than in the packaging workers. While, SOD activities were inversely correlated with AFM_1/Cr levels in packaging workers, and CAT and GPx with AFM_1/Cr levels in packing and grinding workers. So the increase in the levels of AFB_1 and its metabolite AFM_1 increased the levels of free radicals (ROS) and consequently, the DNA oxidative damage biomarker; 8-OHdG besides MDA, while it decreased the levels of antioxidants in erythrocytes and plasma as they were consumed to neutralize the free radicals generated by the toxin. This mechanism was clear among the grinding workers, due to the high levels of AFM_1/Cr. It is also noticed that the highest levels of urinary AFM_1/Cr, oxidative stress biomarkers and antioxidant parameters (except GPx) were among the grinding workers. But in the packaging workers, AFM1/Cr levels were much lower compared to the grinding workers, and there was no significant correlations between AFM_1/Cr and the levels of MDA or 8-OHdG, while, SOD, CAT and GPx were inversely correlated with AFM_1/Cr levels.

Previous studies assessed the effect of exposure to AFB_1 on the levels of MDA and revealed an increase in MDA levels in dose dependent manner in cultured rat hepatocytes (Shen *et al*., 1995), in serum of white rabbits (Prabu *et al*., 2013) and in human lymphocytes cultured in vitro (Aslan *et al*., 2012). They detected increase in MDA levels, while the activities of SOD and GPx decreased upon exposure to AFB_1. Contrarily, the study of Kheir Eldin *et al*. (2008) did not find any significant change in the activity of GPx and levels of DNA and RNA in the hepatic tissue of AFB_1 administered rats. This occurred in spite of the marked increase in the lipid peroxide levels in the same experiment.

Conclusion and recommendations:

Workers in wheat milling industry are at risk of aflatoxin exposure. In the current study, this was evidenced by elevation of levels of aflatoxin M1 in urine, elevation of oxidative stress indicators; MDA and 8-OHdG and decline in the activities of the antioxidant enzymes in erythrocytes and plasma. This attracts the attention for stressing on sound ways of storage and processing conditions during harvest, transport and milling of wheat and other cereal crops. Besides, further investigations are needed to explore the protective effects of natural antioxidant supplementation against AFs hazards in those workers.

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