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Public Health

Oxidative Role of Aflatoxin B1 on the Liver of Wheat Milling Workers

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Abstract

Aim: The study aimed to estimate oxidative role of aflatoxin B1 (AFB1) on the liver in wheat milling workers.

Materials and Methods: Case-control study was conducted to compare between the levels of AFB1/albumin (AFB1/alb), liver enzymes (ALT, AST, GGT, and ALP), P53, MDA, GST, SOD, zinc and vitamin C in 35 wheat milling workers and 40 control subjects.

Results: Statistical analysis revealed that ALT, AST, GGT, ALP, P53, MDA, GST and SOD in workers were significantly elevated compared to their controls. In the milling workers, there were significant correlations between MDA levels and the levels of AST, GGT, and P53, while, P53 was inversely correlated with GST and SOD activities. There were significant correlations between Zn levels and GGT, GST and SOD activities, between vitamin C and GST activities, and vitamin C inversely correlated with MDA.

Conclusion: The present study concluded that the oxidative stress of AFB1 elevated the MDA and the liver enzymes in wheat milling workers. GST has a crucial role in the detoxification of aflatoxin and SOD as a scavenger antioxidant increased in the workers to overcome the oxidative toxic effects of AFB1 on the liver of the workers, and roles of Zn and vitamin C were significant in activation of these processes.

Introduction

Mycotoxins are toxic secondary metabolites produced by species of filamentous fungi growing on foods before harvest and in storage. When ingested, inhaled, or absorbed through the skin, mycotoxins may reduce appetite and cause sickness or death in humans. Aflatoxins are the most important mycotoxins, which are naturally occurring toxins. Aflatoxins consist of a group of approximately 20 related secondary fungal metabolites, although only aflatoxins B_1, B_2, G_1 and G_2 are normally found in foods [1, 2]. They are produced by three species of *Aspergillus*: *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius*, and can occur in a wide range of important raw food commodities including cereals, nuts, spices, figs and dried fruits. These fungi are capable of growing when temperature, relative humidity and product moisture are favorable [3].

Aflatoxin B1 (AFB1) is considered to be among the most toxigenic mycotoxins and can be a significant risk to human and animal health including carcinogenic, mutagenic, teratogenic and immunosuppression. AFB1 has been shown to induce liver cancer (hepatocellular carcinoma or HCC) in many experimental animals [2]. AFB1 has been classified as a group I human carcinogen by the International Agency for Research on Cancer [1]. AFB1 reacts with various liver and blood proteins particularly with serum albumin, to form a stable adduct, which has even been used as a biochemical marker for aflatoxin exposure in human populations. AFB1 is activated mainly by the cytochrome P450 group of enzymes to form the reactive intermediates AFB1-8,9-epoxide [4]. The toxic effects of aflatoxins mostly arise from the binding of this particular epoxide derivative to DNA [5]. Reactive oxygen species (ROS) such as the hydroxyl radical, superoxide anion and hydrogen peroxide are generated as a result of this metabolism, and it is also involved in the toxic

mechanism of AFB1 [6].

Considering the role of ROS in induction of cancers, and the ability of AFB1 to induce oxidative damage to cells and DNA, in addition to the formation of AFB1–DNA adducts, that may play an important role in carcinogenicity of AFB1 [7]. AFB1 epoxides (active metabolites of aflatoxin) react with guanine in DNA, leading to genetic changes where AFB1 frequently induces guanine cytosine to thymine-adenine (G:C to T:A) transversion at the third base in codon 249. Interestingly, mutant DNA in plasma is a biomarker of both AFB1 exposure and potential risk factor for HCC with subsequent P53 mutation [8].

The study aimed to estimate the oxidative role of AFB1 on the liver enzymes and tumor suppressor protein P53 in wheat milling workers, and to find out the role of endogenous antioxidants in minimizing these hazardous effects.

Subjects and Methods

Case-control comparative study was conducted. The study was started by 85 flour milling workers, after exclusion of the workers with positive history of viral hepatitis (HBV or HCV), and exclusion of the workers with low serum levels of AFB1-albumin adduct (AFB1/alb) compared to the control levels. The present study includes two comparative groups; 35 workers with high AFB1-albumin adduct serum levels (workers) and 40 apparent healthy males not occupationally exposed to wheat flour dust (controls), and the control subjects were comparable with the workers in their age, residential area, smoking habits and socioeconomic status.

Random Venous blood samples (5 ml) were collected from all study subjects by sterile disposable syringes. 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. The sera were kept at -20°C for laboratory investigations. The other portion was collected on EDTA for preparing the packed RBCs for determination of SOD.

Aflatoxin B1 (AFB1) was firstly extracted using EASI-EXTRACT® Aflatoxin immune affinity column (Scotland). AFB1 concentrations of the samples were analysed by microtitre plate Enzyme linked immunosorbent assay (ELISA) method using RIDASCREEN® (Germany) [9]. Serum albumin was measured in all serum samples using the Human kits for kinetic determination of Albumin and the AUTOLAB selective access batch auto-analyzer (from Boehringer Mannheim Lab Diagnostics). Serum aminotransferases (ALT and AST) were estimated by the method of Provisional Recommendations on IFCC methods for the measurement of catalytic activities of enzymes [10]. Serum Gamma glutamyl transferase (GGT) was measured using the method of Szasz [11] and serum alkaline phosphate (ALP) was measured by method of Belfield and Goldberg [12].

P53 proteins in serum were determined using the p53 quantitative double-antibody sandwich enzyme-linked immunosorbent assay (ELISA kit) DIACLONE Research protocol (France).

Superoxide dismutase (SOD) activity expressed in U/ml and was measured through spectrophotometer by Biodiagnostic kits, using the method of Nishikimi et al., [13], total glutathione -S-transferase (GST) activity by method of Habig et al., [14]. Lipid peroxidation was estimated in serum as malondialdehyde (MDA) and expressed in nmol/ml, and measured according to Satoh [15].

Zinc (Zn) is chelated by zincon (2-caboxy-2-hydroxy-5-Sulfoformazyl-benzene) at alkaline pH. The formed complex is measured according to Hayakawa and Jap [16]. Vitamin C was measured in serum according to Harris and Ray [17].

The collected data and the laboratory results were computerized. Statistical analysis was done through SPSS version 18. The results are expressed as means ± SD. The statistical significance of the data has been determined using Independent t-test for the comparisons. Correlation coefficient was used to study the relationships between the quantitative data. Significant was considered when P-value ≤ 0.05.

Results

All the included workers and controls in the present study were males. There was no significant difference in the age between the workers and their controls (46.9 ± 8.8 and 42 ± 7.5 years respectively). There was also no significant difference in the smoking habits and smoking index between the two groups (4.9 ± 1.8 and 4.2 ± 1.6 packages/year, respectively). The duration of exposure of the workers were in the range 7-38 years; with average 22.4 ± 10.6 years.

Table 1 shows that the levels of AFB1/Alb, the liver enzymes (ALP, ALT, AST, GGT), and serum P53 of the workers were significantly elevated compared to the control group.

Table 1: Comparisons between the levels of liver enzymes and p53 of wheat milling workers and controls.

	Workers (n = 35)		Controls (n = 40)		Independent t-test	
	Mean	SD	Mean	SD	t-test	P-value
AFB1/Alb (ng/g)	0.09	0.04	0.04	0.01	4.580	P< 0.0001
ALP (IU/L)	96.1	7.34	71.8	21.58	2.929	P< 0.01
ALT (U/L)	31.0	2.70	5.29	1.85	9.431	P< 0.0001
AST (U/L)	45.1	2.50	3.23	1.06	16.682	P< 0.0001
GGT (U/L)	26.6	3.80	6.0	3.67	5.327	P< 0.0001
P53 (Pg/ ml)	2782.3	567.9	1037.9	208.6	2.883	P< 0.01

Table 2 shows that GST, MDA and SOD were significantly increased in the workers compared to the controls group, while, antioxidants vitamin C and levels of Zn in the workers were significantly decreased compared to the controls.

Table 2: Comparisons between the antioxidant enzymes GST and SOD, the oxidative stress marker (MDA), and the levels of Zn and vitamin C in wheat milling workers and controls.

	Workers (35)		Controls (40)		Independent t-test	
	Mean	SD	Mean	SD	t-test	P-value
MDA (nmol/ml)	12.6	2.06	1.5	.21	4.813	P< 0.0001
GST (U/L)	1715.7	317.9	1302.5	251.1	2.698	P= 0.01
SOD (U/ml)	247.4	21.42	194.6	5.11	2.980	P< 0.01
Zn (µmol/l)	101.8	13.58	176.7	64.30	6.287	P< 0.0001
Vitamin C (mg/L)	10.2	1.83	19.4	2.8	4.586	P< 0.0001

In the milling workers with high serum AFB1/Alb levels, there were significant correlations between MDA and the levels of AST, GGT, and P53. While, P53 was inversely correlated with both GST and SOD levels (Table 3).

Table 3: Relationships between the oxidative stress marker (MDA) and the antioxidant enzymes GST and SOD, and the liver enzymes and P53 in the workers.

	MDA (nmol/ml)		GST ((U/L)		SOD (U/ml)	
	r=	P-value	r=	P-value	r=	P-value
ALP (IU/L)	0.3	NS	0.2	NS	0.2	NS
ALT (U/L)	0.3	NS	-0.03	NS	-0.01	NS
AST (U/L)	0.4	P= 0.05	0.02	NS	0.3	NS
GGT (U/L)	0.5	P< 0.05	0.2	NS	-0.1	NS
P53 (pg/ ml)	0.4	P= 0.05	-0.5	P< 0.05	-0.4	P= 0.05

In the milling workers with high serum AFB1/Alb levels, there were significant correlations between Zn levels and the activity of GGT, GST and SOD. Also, there was significant correlation between vitamin C and GST, while MDA was inversely correlated with vitamin C (Table 4).

Table 4: Relationships between Zn and vitamin C levels and the liver enzymes, P53, MDA and the antioxidant enzymes GST and SOD in the workers.

	Zn (µ mol/l)		Vitamin C (mg / L)	
	r=	P-value	r=	P-value
ALP (IU/L)	0.1	NS	-0.1	NS
ALT (U/L)	0.1	NS	-0.3	NS
AST (U/L)	0.2	NS	-0.04	NS
GGT (U/L)	0.6	P< 0.005	-0.1	NS
P53 (Pg/ ml)	-0.2	NS	-0.2	NS
MDA (n mol/ ml)	0.2	NS	-0.5	P< 0.05
GST (U / L)	0.4	P= 0.05	0.4	P= 0.05
SOD (U/ml)	0.4	P= 0.05	0.2	NS

Discussion

Only a few reports are available on the effects of occupational exposure to fungi in grain transformation industry. In previous studies, chronic environmental exposure to the high concentrations of *Aspergillus flavus* caused significant elevation in the urinary levels of AFM1 [19], and in the blood levels of AFB1 [20]. The wheat milling workers with high serum AFB1/Alb levels detected in the previous study of Saad-Hussein et al. [20] were included in the current study. The present study confirmed that the levels of AFB1/Alb were significantly higher in the included workers compared to their controls.

Hepatotoxicity induced by AFB1 is characterized by periportal hepatocellular necrosis, bile duct injury, and hemorrhage in pathology [21]. Since the liver enzymes (ALT, AST, ALP and GGT) are released from the liver into the blood stream when the liver tissues are injured, their activities in serum are critical diagnostic indicators for determining the extent of hepatic damage caused by AFB1 [6].

The present study revealed that there was significant elevation in the liver enzymes (ALT, AST, ALP and GGT) of examined milling workers compared to their control. Experimentally, the activities of ALT and AST in aflatoxin-treated animals were significantly higher compared to the controls 22]. Elevated levels of both ALP and GGT are suggestive of liver or bile duct disease, and GGT is a prime marker of bile duct epithelial proliferation that is typical of aflatoxicosis [23].

Similarly, in the previous study, the liver enzymes AST and ALT of workers handling wheat flour were found to be significantly elevated compared to the controls, and these elevations were significantly increased with the increase in the environmental exposures to *Aspergillus flavus* [20]. But in contrast to the present study, Saad-Hussein et al. [20] revealed that there was no significant difference in ALP levels between the workers and the controls. This could be attributed to the inclusion of all the milling workers with high and low levels of serum AFB1/alb in the study, while, in the present study only milling workers with high serum levels of AFB1/alb were included. The extent of damage due to AFB1 however differs and depends on the dose, duration of exposure and route of administration of the toxin, and may range from acute to chronic, eliciting a carcinogenic response with long term chronic exposure [22]. Also experimentally, Fu et al. [24] found that there were no effects of AFB1 on ALT, AST, ALP and GGT, and they attributed that to the short duration of exposure (42 days).

The levels of P53 in normal cells are low, caused by a short half-life of the protein. However, P53 activation is induced by a number of stress signals, including oxidative stress, DNA damage, and activated oncogenes. The oxidative damage due to AFB1 exposure might induce activation of P53 [25]. Tong et al. [26] mentioned that P53 mutations may be a late event that promotes carcinoma development in the liver. Thus, the significant elevation of the serum P53 in workers in the current study could not be neglected, and tumor markers must be done and the health status of exposed workers must be followed.

However, the mechanisms of ROS generation induced by AFB1 have not been completely elucidated [6, 8]. One of the manifestations of AFB_1-induced toxicity is aflatoxicosis, which has been associated with enhanced ROS generation and oxidative stress [27]. Previous researches revealed that the effect of exposure to AFB1 on the levels of MDA is a dose dependent manner in human lymphocytes cultured in vitro [28], and in wheat milling workers [29]. The urinary AFM1; the metabolite of AFB1, was significantly higher in the grinding workers compared with the workers in the other departments

of the mill manufacture, and MDA was proved to be correlated with the AFM1 in the grinding workers, while this relationship was not detected in the workers in the other departments [29]. In the present study, MDA was significantly elevated in the wheat milling workers with high serum levels of AFB1/alb compared to the controls. The increased production of MDA; as a late biomarker of oxidative stress regardless of the way it is induced, could explain the dose-dependent high cytotoxic and genotoxic potential of AFB1 [30]. It was reported that AFB1 generates ROS that has an important contribution in the hepatic damaging process [7], and that could explain the significant correlation between MDA and the liver enzymes AST, GGT and P53 detected in the present study.

The levels of enzymatic and non-enzymatic antioxidants are the main determinants of the antioxidant defense mechanism of the cell. They can act synergistically as a scavenge of ROS and prevent lipid peroxidation. The lipid peroxidation is proportionally increased by elevating the generation of ROS with an attendant decrease in primary antioxidant status [7]. But, the present results revealed that the enzymatic antioxidants GST and SOD in the examined workers were significantly elevated in comparison to their controls.

The significant elevation in GST of the workers compared to the controls in the present study could be explained by quick release of GST in large quantities into the blood stream during hepatocellular injury due to the high AFB1 levels. In humans and most animals, the principal route of AFB1 detoxification appears to be through conjugation with endogenous GSH, a reaction catalyzed by GST, it represents the most important detoxification system, GST plays a key role in the protection of cells from AFB1 toxicity because conjugation of the electrophilic AFB_1-8,9-epoxide with GSH is an alternative fate to binding to nucleophilic centers in cellular macromolecules [5]. Induction of GST synthesis is a protective mechanism that occurs in response to xenobiotic exposure, and this could explain the significant elevation of GST among the milling workers compared to the controls in the present study. Similarly to the present results, Borroz et al. [31] found that mice are resistant to the carcinogenic effects of aflatoxin and this resistance results in increase expression of an isoenzyme of GST with high activity toward AFB,-8,9-epoxide.

The antioxidant enzyme SOD plays an important role in the elimination of ROS derived from the peroxidative process, SOD removes superoxide by converting it to H_2O_2 [27], and similarly, this could also explain the significant elevation of SOD of the milling workers compared to their controls in the current research. Contrary to the current result, Alpsoy et al. [30] found that in human lymphocytes, AFB1 significantly decreased the activities of SOD. While, Huang et al. [32] showed that, the activities of SOD were not significantly different in the AFB1 exposed groups compared to the controls. These discrepancies might be due to difference in the concentrations and the uptake route of AFB1.

The current result revealed that P53 was significantly correlated with MDA and inversely correlated with GST and SOD. P53 itself is redox active due to the presence of cysteines that contain redox sensitive thiol groups (-SH). In human P53, there are two clusters of cysteines in the DNA-binding domain, which are essential to the specific binding of P53 to its consensus sequence. S-glutathionylation of P53 occurs both in vitro and in vivo, and is regulated by the ratio of GSH/GSSH [33]. The principal route of AFB1 detoxification appears to be through conjugation with endogenous GSH, a reaction catalyzed by GST [5], and the levels of total intracellular GSH are lowered during the generation of GSH–*S*-conjugates by GST [34].

Vitamin C, which includes ascorbic acid and its oxidation product-dehydroascorbic acid, has many biological activities in human body. It can be defined as an enzyme cofactor, a radical scavenger, and as a donor/acceptor in electron transport at the plasma membrane [35]. Vitamins C were shown to play a great role in reducing the oxidative stress induced by aflatoxins including AFB1 [8].

Vitamin C is an important non-enzymatic antioxidant. In the present study, vitamin C was significantly reduced in the milling workers compared to their controls. In agreement with the current results, Kanchana et al. [27] found a significant reduction in vitamin C levels in AFB1-administered rat, and they defined this as a responsible for increased lipid peroxidation which resulting in oxidative stress observed during aflatoxicosis.

Ascorbic acid is known to affect the activities of several liver enzymes [36] and has an important role in maintaining reduced glutathione levels [37]. However, vitamin C was not correlated with the liver enzymes in the present study among the milling workers; it was significantly correlated with GST and inversely correlated with MDA. According to that, vitamin C seemed to be an important antioxidant for protection against the hepatic damaging effects of aflatoxins.

Zinc (Zn) is considered as one of the most sensitive element to intoxication with aflatoxin, since its value decreased during the clearance period of the effect of aflatoxicosis in growing lambs [38]. The role of Zn is very important in the antioxidant defense mechanism, as well as in regeneration of damaged cells [39], because it is a constitutive element of SOD (CuZnSOD) [40]. Experimentally, Zn deficiency in rats displayed significant decreases in the blood and liver of SOD activity [41].

In the present study, serum Zn had been significantly reduced in the milling workers compared

to their controls, and was significantly correlated with the liver enzyme GGT. Similarly Abd El Moety et al. [42] found that GGT was positively correlated with Zn in patients with positive hepatitis C. Cellular GGT is a membrane bound enzyme that transfers the glutamyl moiety of glutathione to acceptors. Its main function is to make cysteine available for synthesis of glutathione within the cell, thus preventing oxidative stress [43]. Also, Jagadeesan [44] reported that the activity of GST a key enzyme in conjugation reaction was significantly lowered in Zn deficiency. This could explain the significant correlation between the serum Zn levels in the workers in the present study with the antioxidants GST, as well as with SOD. Therefore, the importance of Zn in protection of the human against aflatoxin is considered, due to the crucial role of glutathione and GST in the detoxification of aflatoxin and the antioxidant role of SOD.

In conclusion, the present study revealed that the oxidative stress of AFB1 elevated the MDA and the liver enzymes in wheat milling workers. GST has a crucial role in the detoxification of aflatoxin and SOD as a scavenger antioxidant increased in the workers to overcome the oxidative toxic effects of AFB1 on the liver of the workers, and roles of Zn and vitamin C were significant in activation of these processes.

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