ORIGINAL ARTICLES

Hepatotoxic Effect of Aflatoxin B1 and Polymorphisms in Glutathione S-Transferase in Millers Occupationally Exposed to Aspergillus

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ABSTRACT

Little is known whether occupational exposure to aflatoxin might have a potential hazard. The present work aimed to study the hepatotoxic effects of occupational exposure to AFB1 as a metabolite of high Aspergillus concentrations in the working environments, and the polymorphism of GST gene in exposed workers. The study was performed on 97 flour mill workers exposed to high Aspergillus concentrations and 78 non-exposed controls. The levels of AFB1/Alb (ng/g), AST and ALT of the workers were significantly higher than the controls, while there was no significance difference in the ALP levels between the two groups. The present results revealed that AFB1/Alb levels were significantly higher in the workers with the different GST alleles compared to the control groups. Additionally, AFB1/Alb levels were significantly higher in the workers with GSTT1 compared to the workers with different GST alleles (GSTM1 and Null (GSTM1&GSTM1)) and the controls with Null (GSTM1&GSTM1) alleles. In conclusion, occupational exposure to the high concentrations of Aspergillus in the workplace may cause an increase in the AFB1/Alb and the liver enzymes in flour mill workers. These results also showed that the Null (GSTM1 & GSTT1) alleles are the most common type in the studied population. The workers with GSTT1 have lower ability to detoxify AFB1.

Key words: Millers; aflatoxin B1; liver enzymes; GSTM1; GSTT1.

Introduction

Mycotoxins are secondary metabolites of different fungal species, and it have high cytotoxic potential (Bernabucci et al., 2011). The greatest significant food-borne mycotoxins in Africa and tropical developing countries are likely to be aflatoxins and fumonisins (Ilesanmi and Ilesanmi 2011). Aflatoxins are the most toxic group of mycotoxins that are produced by some Aspergillus species (Aspergillus flavus, Aspergillus parasiticus and the rare Aspergillus nomius) (Park et al., 2012). Although AFB1 is a well studied mycotoxin, it is not known whether exposure to AFB1 containing dust poses a health risk to the exposed workers in the indoor environments or not (Liao and Chen 2005).

The International Cancer Research Institute identifies aflatoxins (AFs) as class 1 carcinogens (Asim et al., 2011 and Prabu et al., 2013). AFB1 is the most potent genotoxic and carcinogenic form of AFs (Park et al., 2012). The carcinogenicity of AFB1 is partly ascribed to its ability to induce DNA damage (adduct) and mutagenesis (Besaratinia et al., 2009). AFB1 is bioactivated through epoxidation of the terminal furan ring double bond by cytochrome P450 (CYP450) enzymes generating the electrophilic intermediate AFB1-8,9-epoxide,(AFBO) (Yang et al., 2012). AFB1 exo-8,9-epoxide is considered as an active electrophilic form of aflatoxin which may attack nitrogen, oxygen and neutrophilic sulphur that exist in different cellular components (Khoshpey et al., 2011), and can further bind covalently to cellular macromolecules such as DNA, RNA, and proteins (Gross-Steinmeyer and Eaton 2012). It intercalates between the bases in DNA, and form covalent bonds with DNA by electrophilic attack on the N7 position in guanines, leading to the formation of the adduct 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AFB1-N7-Gua) (Gous et al., 2009). AFB–DNA-adducts, if not removed by DNA repair enzymes, have the potential to develop into somatic alterations if they are localized in transcriptional active DNA regions (Gross-Steinmeyer and Eaton 2012).

The genotoxic AFBO may be trapped by glutathione S-transferases (GST), thereby protecting DNA and proteins from adduction. The AFBO-GSH conjugate is eventually excreted as AFBO-mercapturic acid in the urine. Conjugation of AFBO with reduced glutathione serves as a critical detoxification pathway (Gross-Steinmeyer and Eaton 2012). The GST genes are highly polymorphic and frequently inducible (White et al., 2007). In humans, seven distinct gene families encode soluble GSTs; the glutathione S-transferases (GST) expressed in human tissue comprise the alpha (A), mu (M), pi (P), theta (T), kappa (K), omega (O) and zeta (Z)
gene families (Yu et al., 2011 and Uzunoğlu et al., 2006). Among them, four are mainly expressed in human tissues: GSTA, GSTM, GSTT, and GSTP (Wang et al., 2010).

GSTM1, GSTT1, and GSTP1 genes have been extensively examined in association with risk of cancer and clinical outcomes of cancer patients (Yu et al., 2011). Both GSTM1 and GSTT1 enzymes are known to catalyze the detoxification of reactive oxygen and lipid peroxidation products (Uzunoğlu et al., 2006). Polymorphic deletion variants in the GSTM1 and GSTT1 genes produce either a functional enzyme (non-deletion alleles or heterozygous deletion, GSTM11 and GSTT11) or result in the complete absence of the enzyme (homozygous deletion alleles, GSTM1null and GSTT1null) (Xiao and Ma 2012). In our work, we studied the hepatotoxic effects of occupational exposure to aflatoxin as a metabolite of the high concentrations of Aspergillus in the working environments, and Polymorphisms in Glutathione S-Transferase gene (GSTM1 and GSTT1) in the exposed millers.

Methodology:

Subjects:

This is a cross-sectional comparative study. This study was performed on 97 flour mill workers (71 males and 26 females) from flour mill factory in Helwan city, Egypt. All included workers were exposed for at least 5 years to high environmental concentration of Aspergillus flavus and its mycotoxin (aflatoxin) in their workplaces as reported in the Saad-Hussein Project (2010-2013). Other 78 healthy subjects of the same socioeconomic status (56 male and 22 female) were included in the study as control group not occupationally exposed to Aspergillus flavus. The control group was matched for their socioeconomic status, age and smoking habits. Ethical consent was taken from the Ethical Committee of the National Research Center (NRC), and written consent was obtained from all the included subjects.

Biological Samples:

About 5 ml blood sample was collected from each participant and divided in 2 tubes, 2 ml blood on EDTA tube which was kept frozen at -20°C for screening of glutathione-S-transferase (GST) polymorphisms. And 3 ml in another dry tube which was left for coagulation at the room temperature for 30 min, then centrifuged at 3000 rpm for 10 minutes. The separated serum was divided into several aliquots and kept frozen at-20°C for the determination of serum aflatoxin B1 level (AFB1) and liver enzymes activity.

Laboratory investigations:

Measurement of Aflatoxin–albumin adduct level (AFB1/Alb):

a. Determination of Aflatoxin B1 (AFB1) level in serum:

Aflatoxin B1 was firstly extracted from the serum using EASI-EXTRACT® Aflatoxin immunoaffinity column (Scotland). The extracted sample was applied to the ELISA according to RIDASCREEN® Aflatoxin B1 ELISA kit.

b. Serum Albumin (Alb):

Serum albumin was determined by colorimetric method according to (Doumas and Biggs 1976).

Measurement of serum enzyme levels:

Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined according to the method of Reitman and Frankel (1957). Serum alkaline phosphatase activity (ALP) was determined by colorimetric method according to Belfield and Goldberg (1971).

Screening of glutathione-S-transferase (GST) polymorphisms (GSTM1, GSTT1 genotypes) using polymerase chain reaction PCR:

A. DNA extractions:

DNA was extracted from whole blood sample using Genomic DNA Purification kit (Gene JET™/Fermentas).
B. PCR amplification:

The GSTM1 and GSTT1 genotypes were determined by co-amplification of both genes \((Luo \textit{et al}., 2005)\) with PCR. Briefly, PCR was performed in a 25-ml mixture containing the buffer supplied by Promega (Madison, WI, USA), 250 ng genomic DNA, Taq DNA polymerase (1 U), four bases (dNTP) and 200 µg of each primer. The primers used for the GSTM1 gene were 5’ -CTGCCCTACTTGATTGATGGG-3’ and 5’ -CTGGATTGTAGCATGATGC-3’. The primers used for the GSTT1 gene were 5’ -TTCTTAC TGGTCCTCACATCTC-3’ and 5’ -TCACC-GGATCATGGCCA GCA-3’. The human B-globin gene (110 bp) was also amplified in each reaction as a positive control to confirm the presence of amplifiable DNA in the samples.

The primers used for B-globin were 5’ -ACACAACT GT GTTCACTAGC-3’ and 5’ -CAACTCATCCACGTTCACC-3’.

The amplification was carried out in 35 cycles with denaturation at 94°C for 1 min 30 s, annealing at 52°C for 1 min, and extension at 65°C for 1 min. The PCR products were then resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide and photographed under ultraviolet light. Individuals with one or more GSTM1 alleles had a 273-bp fragment, and individuals with one or more GSTT1 alleles had a 480-bp fragment.

Statistical Methodology:

Statistical analysis was done through SPSS version 18.0. Quantitative data was represented in form of mean ± standard deviation (SD), and Qualitative data in form of number (No.) and percent (%). Independent t-test was used for comparing the quantitative results between two groups. Chi-square \((\chi^2)\) were used for analysis of qualitative data, and likelihood ratio was used if more than 25% of the cells have expected count less than 5. Analysis of Variance (ANOVA) and least significant difference (LSD) were used in the analysis to compare quantitative results between more than two groups. Correlation coefficient was used to study the relationship between two quantitative variables. The level of significant was considered at \(P < 0.05\).

Results:

Table 1 showed that there was no significant difference between the workers and the controls in their age, smoking index. High significant elevations were detected in the AFB1/Alb levels, AST and ALT activity of the workers compared to their controls, while there was no significance difference in the ALP activity between the two groups

<table>
<thead>
<tr>
<th></th>
<th>Workers (97)</th>
<th>Controls (78)</th>
<th>Independent t-test</th>
<th>(t)-test</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Mean 46.9 SD 7.57</td>
<td>Mean 48.7 SD 7.95</td>
<td>1.49</td>
<td>0.137</td>
<td></td>
</tr>
<tr>
<td>Smoking index SI (Package/year)</td>
<td>6.58 SD 11.14</td>
<td>5.22 SD 8.3</td>
<td>0.9</td>
<td>0.388</td>
<td></td>
</tr>
<tr>
<td>AFB1/Alb (ng/g)</td>
<td>0.06 SD 0.04</td>
<td>0.04 SD 0.01</td>
<td>5.58</td>
<td>(&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>26.1 SD 8.30</td>
<td>16.2 SD 10.9</td>
<td>6.334</td>
<td>(&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>36.7 SD 1.06</td>
<td>21.7 SD 2.13</td>
<td>6.308</td>
<td>(&lt;0.0001)</td>
<td></td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>80.2 SD 25.43</td>
<td>76.1 SD 23.96</td>
<td>1.05</td>
<td>0.294</td>
<td></td>
</tr>
</tbody>
</table>

Homzygous deletion alleles (Null GSTM1& GSTT1) were detected in 100% of the control subjects and in 72.2% of the millers. Figure (1) showed that there was a significant difference in the GST gene polymorphism between the two groups.

The PCR results for GST polymorphism showed the GSTT1 allele at 480bp and GSTM1 allele at 273bp. The absence of GSTT1 was expressed as Null GSTT1 and the absence of GSTM1 was expressed as Null GSTM1. Null GSTT1&GSTM1 indicated the absence of both GSTT1 and GSTM1 alleles (figure 2).

The levels of AFB1/Alb ratio was significantly higher in the workers with GSTT1 allele compared to the controls and to the workers with the other types of GST alleles. Also, the levels AFB1/Alb ratio of the workers with GSTM1 allele and those with Null (GSTM1&GSTT1) allele were significantly higher compared to the controls (Table 2). While no significant differences in levels of AFB1/Alb ratio were detected between workers with GSTM1 allele and controls.
Fig. 1: Distribution of the GST gene polymorphism among the worker and the control groups

Table 2: Comparison between the AFB1/Alb levels according to the GST gene polymorphisms in the workers and their controls

<table>
<thead>
<tr>
<th>GST gene</th>
<th>Workers</th>
<th></th>
<th></th>
<th>Anova</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Null GSTT1 &amp; GSTM1 (70)</td>
<td>0.06</td>
<td>0.03</td>
<td>LSD</td>
<td>(T1, controls)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST M1 allele (19)</td>
<td>0.06</td>
<td>0.04</td>
<td>LSD</td>
<td>(T1, controls)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST T1 allele (6)</td>
<td>0.08</td>
<td>0.04</td>
<td>LSD</td>
<td>(Null workers, M1, T1&amp;M1, controls)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GS M1 &amp; GST T1 alleles (2)</td>
<td>0.04</td>
<td>0.03</td>
<td>LSD</td>
<td>(T1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Null GSTT1 &amp; GSTM1 (78)</td>
<td>0.04</td>
<td>0.01</td>
<td>LSD</td>
<td>(Null workers, M1, T1)</td>
<td></td>
</tr>
</tbody>
</table>

NB: T1= GST T1, M1= GST M1, Null workers= Null GSTT1 & GSTM1

Table (3) found that there was significant increase in the AST and ALT activities of the workers with the different GST gene polymorphism compared to the control group. While, there was no significant difference in ALP activity between the workers with the different GST gene polymorphism compared to the control group.
Table 3: Comparison between the liver functions of the workers with different GST gene polymorphism and their controls

<table>
<thead>
<tr>
<th>GST</th>
<th>Mean</th>
<th>SD</th>
<th>ANOVA</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Null GSTT1&amp;GSTM1 (70)</td>
<td>26.22</td>
<td>9.28</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>GST M1 allele (19)</td>
<td>25.39</td>
<td>4.71</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST T1 allele (6)</td>
<td>27.67</td>
<td>6.41</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM1 &amp; GSTT1 alleles (2)</td>
<td>22.50</td>
<td>2.12</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Null GSTT1&amp;GSTM1 (78)</td>
<td>16.2</td>
<td>1.30</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST M1 allele (19)</td>
<td>33.7</td>
<td>7.79</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST T1 allele (6)</td>
<td>33.67</td>
<td>7.80</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM1 &amp; GSTT1 alleles (2)</td>
<td>30.5</td>
<td>2.12</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>Null GSTT1&amp;GSTM1 (70)</td>
<td>37.8</td>
<td>10.48</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST M1 allele (19)</td>
<td>33.7</td>
<td>7.79</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST T1 allele (6)</td>
<td>33.67</td>
<td>7.80</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM1 &amp; GSTT1 alleles (2)</td>
<td>30.5</td>
<td>2.12</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Null GSTT1&amp;GSTM1 (78)</td>
<td>21.7</td>
<td>2.13</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>ALP (IU/L)</td>
<td>Null GSTT1&amp;GSTM1 (70)</td>
<td>75.6</td>
<td>25.89</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST M1 allele (19)</td>
<td>77.17</td>
<td>16.90</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GST T1 allele (6)</td>
<td>80.50</td>
<td>24.73</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSTM1 &amp; GSTT1 alleles (2)</td>
<td>71.0</td>
<td>26.87</td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>Null GSTT1&amp;GSTM1 (78)</td>
<td>76.1</td>
<td>23.96</td>
<td>C</td>
<td></td>
</tr>
</tbody>
</table>

C = Control

Discussion:

Airborne AFSs in agricultural food production have been studied extensively. Occupational exposure to organic dusts commonly contaminated with fungi and their mycotoxins AFSs; such as flour and cotton dust, may induce numerous symptoms and diseases (Saad-Hussein et al., 2012). Workers in the agricultural food industries are at high risk of ingestion, transmucosal absorption, and inhalation of AFB1 released during product preparation or processing (Yang et al., 2012). A project by Saad-Hussein (2011-2013) revealed that high concentration of airborne fungal communities were isolated from the work environment of flour mill factory in Helwan city. Genus Aspergillus was predominant and among the Aspergillus species, A. flavus was the most commonly isolated species. According to that, the flour mill workers are occupationally exposed to airborne A. flavus strains producing AFSs.

Gouas et al. (2009) mentioned that AFB1/Alb levels in peripheral blood are a reliable indicator of long-term exposure to aflatoxin. Van Vleet et al., (2001) have detected high AFB1 concentrations (52 ppm) in respirable dusts generated in many of the steps of grain harvest and processing. In accordance to our study AFB1/Alb levels were measured in the two studied groups as an indicator of chronic exposure to AFB1. Although the workers and their controls were matched for their socioeconomic status and dietary habits, there was a significant elevation of aflatoxin–albumin adduct level (AFB1/Alb) in the workers compared to their controls. This could be attributed to an extra source of exposure to aflatoxins production in their workplace.

Liver is known to be the target organ following ingestion of AFSs. It is well known that AFSs are hepatotoxic, hepatocarcinogenic, mutagenic and immunosuppressive (Oguz et al., 2003). Liao and Chen (2005) found a high concentration of the carcinogen AFB1 in respirable, airborne dust, and considered AFB1 a risk factor for occupational carcinogenesis. In our study there was a significant elevation in the levels of AST and ALT enzymes of the workers compared to their controls. The increased levels of AFB1/Alb and liver-specific biochemical variables was reported in many animal studies (Yousef et al., 2003 and Barton et al., 2000), but not by (Cheng et al., 2001). Such elevation in liver enzymes suggests either hepatic parenchymal cell injury (ALT, AST) or biliary tract alterations (ALP). Francis et al. (2010) and Tao et al. (2005) detected an increase in AFB1/Alb levels and liver enzymes. They suggested that aflatoxin induced injury to both hepatic parenchyma and biliary tract.

In this study the effect of GST polymorphism was studied in the flour mill workers and their controls relative to its two genotypes GSTM1 and GSTT1. All controls have null GSTT1&GSTM1 genotypes, while in workers; the genotypes have been identified with different frequencies. About 72% of the workers had null GSTT1&GSTM1, and the other workers had GSTM1 allele (19.6%), GSTT1 allele (6.2%) and GSTT1&GSTM1 alleles (2.1%).

The present results revealed that AFB1/Alb levels were significantly higher in the workers with GSTT1 allele compared to the workers with other allele types and to the control. Additionally, AFB1/Alb levels were significantly higher in the workers with GSTT1 allele compared to the workers with other allele types and to the controls.

Moreover, the interaction terms between serum AFB1/alb adducts level and workers with (GSTM1 &GSTT1) allele was at marginal statistical significance levels, possibly resulting from the small sample size in this study.

Also the subjects with Null GSTM1&GSTT1 were having the ability to detoxify aflatoxin even though they didn’t possess any of GSTM1 or GSTT1 active allele form. The presence of Null GSTM1&GSTT1 alleles with the highest frequency among control subjects (100%) and flour mill workers (72%) supported the idea that these
entire individual couldn’t have a non functional Null GST allele especially with their ability to detoxify aflatoxin. They may have another GST allele which detoxifies the AFs significantly higher than GSTM1 or GSTT1. In our study all the detected GST genotypes were able to detoxify aflatoxin with different degrees. On the contrary Gross-Steinmeyer and Eaton (2012) who suggested that the GSTM1 genotype was the only human GST with measurable catalytic activity toward AFBO. According to Chen et al. (2012) subjects with GSTT1 null genotype may be genetically predisposed for increased cancer risk.

In conclusion, occupational exposure to the high concentrations of Aspergillus in the workplace may cause an increase in the AFB1/Alb and the liver enzymes in flour mill workers. These results also showed that the Null GSTM1 & GSTT1 genotype is the most common type in the studied population. All subjects with null GSTT1 &GSTM1 have higher ability to detoxify AFB1, while those with GSTT1 have lower ability to detoxify AFB1. Further studies are needed to identify this mechanism.

Recommendation:

It’s necessary to provide safety clothes in form of protective mask and gloves to the flour milling workers for their safety; especially the workers with GSTT1 allele. Improving the ventilation of the workplace is mandatory.

References


