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Evaluate the Possible Roles of *GSTM* and *GSTT* Genes Polymorphism and its Interaction with PAHs in Charcoal Exposed Workers

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ABSTRACT

Deletion of the polymorphism of GST genes accompanied several kinds of cancers such as skin, lung, and colon cancers. The GSTs genetic polymorphisms possibly have a great effect on susceptibility of individual to "damage of DNA" and the occurrence of different types of cancers. This study aimed to detect the genetic damage in population who working in charcoal-making kilns by using the alkaline comet assay. Blood PAHs concentrations in blood and the effect of the glutathione S-transferases genotype polymorphisms on the DNA damage were studied. The values of the mean tail moment (TM) in exposed subjects were significantly higher (14.12±0.65; $p<0.05$) than control (0.31±0.23; $P<0.05$). The results demonstrate a considerable relationship between the TM values and GSTM1 and GSTT1 genotypes that recorded in this study. The results suggested that the DNA damage in charcoal-making workers were increased with the increase the exposure time to PAHs. We concluded that the GSTM1 and GSTT1 genes polymorphisms may be responsible for the observed DNA damage.

Keywords: Glutathione S- Transferases, Genes Polymorphism, Genotoxicity, Polyaromatic hydrocarbons, Charcoal factory.

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INTROSUCTION

Charcoal production provides wealth and Job opportunities to the residents, but at the same time for decades consider a serious threat to the environment and living things. The health and environmental problems are associated with nature and types of heavy metals, methods of operations, and the nature of geological area covered[1]. Charcoal is an end-product of wood carbonization or " controlled conditions of pyrolysis" in a closed space such as a charcoal kiln. Control is a process of chemical decomposing to form charcoal by preventing the entry of air during the carbonisation or pyrolysis to stop the wood from burning away to ashes[2]. The main cause of organic aerosols is the combustion of solid material. Polycyclic aromatic hydrocarbons (PAHs) in these aerosols are of the key interest of toxicants agents due to their mutagenicity and carcinogenicity[3]. PAHs are the most common pollutants in the environment involved in carcinogenicity[4]. These compounds have three aromatic rings or more in their structures and the most PAHs spreadable in the environment are naphthalene, phenanthrene, Benz(a)pyrene and anthracite. It results from the combustion of fossil fuel as byproducts of industrial, and natural emission[5,6]. Coal production, natural gases, crude oils, heavy and light metals production are the main sources of the PAHs by which they enter the environment. There are more than 100 types of PAHs exist in the environment. However, only (6 to16) are public health and environmentally monitored and analyzed[7]. PAHs accumulation may cause an intense effect on public health because they are teratogenic, mutagenic and toxic. PAHs biotransformation by CYP 450 system enzymes like CYT family and microsomal epoxide hydrolase leads to the formation of free radicals such as reactive oxygen species. Consequently, the latter causes DNA adducts due to binding to DNA chemically[8]. Furthermore, some reports recorded that the PAHs metabolites effect on proteins and lipids structures and some of halogenated PAHs may pass the placenta and cause fetal deformity[9].

Various polar compounds are produced in the second phase of xenobiotic biotransformation[10] by conjugation with (eg; with glutathione or glucuronic acid) and thereby become less toxic and excreted out of the body by different excretory pathways. This major reaction is facilitated by different transferases such as SULT, UGT, GST, and N-acetyltransferases (NAT)[11,12]. GSTs have three types: mitochondrial, cytosolic, and membrane-bound microsomal[13]. The superfamily of cytosolic GSTs is existed commonly in the liver and also found in other types of body tissues including various isoenzymes. The glutathione is the most common "cytosolic nucleophilic tripeptide" (CNT) that bind to epoxides and free radicals by the action of GSTs. Subsequently, it decreases the formation of oxidative stress[14]. Besides, the main cyctolic enzymes superfamilies are GSTM1 and GSTT1[15] which are encoded by *"GSTM1 and GSTT1 genes*" and the main function of GSTM1 is the detoxification of CYP1A1 products and the toxic xenobiotics such as the environmental pollutants[16]. While the conjugation of oxidized lipids and halogenated compounds is facilitated by GSTT1. Conjugation with glutathione considers as a safe and active reaction to remove the toxins preventing cells from oxidative stress, DNA damage, and mutation[17]. The two members of the GST family "μ (GSTM1) and θ (GSTT1)" are the most common show deletion polymorphism. The absolute absence of both alleles or one allele resulting in complete deficiency or reduction in

enzyme activity, respectively[18-20]. Susceptibility of an individual to carcinogenesis and the inflammation may be accelerated or decelerated by Genetic polymorphisms[21]. The study of interactions between environmental pollutants and genes will give an insight into a great understanding of the pathogenic mechanism of several diseases. In this case, this research focus on the exposure to pollutants like PAHs and the role of GSTM1 and GSTT1 gene polymorphism in charcoal making workers.

MATERIAL AND METHODS

1. Blood collection:

About (3) ml of blood was collected during a period of May/2019 to January/2020 from (200) exposed and (100) control individuals. Samples kept in Heparinized tubes. All samples were labeled accurately and transported immediately in an icebox to the lab of the Material Research Department at the Ministry of Science and Technology, Iraq for PAHs analysis. The research protocol complied with the ethical principles and approved by the Ethics in Research Committee of Baghdad University.

2. Detection of Polycyclic hydrocarbons in serum by (RP-HPLC): PAHs were extracted from the blood sample based on the method of Van Schooten et a[22]. Before the HPLC analysis. initially, the n-hexane was used in liquid-liquid extraction step and then changed to acetonitrile. Clean up process was performed using SPE cartridges conditioned with acetonitrile. The final samples were analyzed on reversed-phase high-performance liquid chromatography (RP-HPLC) (Shimadzu 760 A. flameless, Japan-koyota). C-18 ODS (75x4.6mm I.D., particle size 3.5mm) analytical column with a pre-column from Waters (Water Milford, USA). Acetonitrile (A) and water (B) were used as a composition of the mobile phase. Detection program and elution conditions were carried out according to the methods of Barranco et al[23] as follow:

1. 0–10 min was the time for elution conditions.
2. The A isocratic was (50) %: time (1-10 min)
3. The linear gradient 50 percent A-100 percent A; (10–24 min)
4. The "A isocratic" (100%): (24–35 min)
5. The "flow rate" was 1ml/60 seconds
6. The "injection volume" was 20 µl.
7. Recoveries were measured by spiked and observed levels of PAHs (78-94) %.

3. GSTM1 and GSTT1 genotyping

DNA extraction:

DNA was isolated from 100µl of whole blood using (Quick-gDNA™ Blood MiniPrep) kit (Zymo/USA). In total, DNA of 100 cases and 50 controls were available for genotyping the DNA.

The electrophoresis:

Electrophoresis is a procedure used for determination of the DNA following the extraction or for detection the results of interaction of polymerase chian reaction (PCR) on agarose gel (Conda / USA). The gel was examined at UV (336) nm after putting it in the pool containing (30µl) Red safe stian (Intron / Korea) and (500 ml) distill water for detecting the DNA in agarose gels.

The primers:

The primers were provided by the Integrated DNA Technologies company (IDT, Canada). Primers dissolved in the sterile DW to prepare a stock solution with total concentration 100pmol/µl and then stored at (-20). A 10pmol/µl as working primer were prepared by dissolving 10µl of the stock solution in (90µl) DW to give the final volume was (100) µl. **Tables 1, 2, and 3.**

Maxime PCR PreMix kit:

INtRON'sMaximePCR-PreMix Kit was used in our study. MaximePCR Pre Mix Kit is summarized in **table 4**. While the optimum condition of detection GSTM1 and GSTT1 genes were summarized in **table 5**.

Table 1: The specific primer of GSTM1gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- GAA CTC CCT GAA AAG CTA AAG C- 3'	54	45.5	215bp
Reverse	5'- GTT GGG CTC AAA TAT ACG GTG G- 3’	55.9	50	

Table 2: The specific primer of GSTT1gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- TTC CTT ACT GGT CCT CAC ATC TC- 3’	55.8	47.8	480bp
Reverse	5'-TCA CCG GAT CAT GGC CAG CA - 3’	61.7	60	

Table 3: The specific primer of B-GLOBIN gene

Primer	Sequence	Tm (°C)	GC (%)	Product size
Forward	5'- CAA CTT CAT CCA CGT TCA CC- 3'	53.9	50	267bp
Reverse	5'- AAG AGC CAA GGA CAG GTA C- 3'	54.1	52.6	

Table 4: Mixture of the specific interaction for diagnosis gene

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1µl)
Reverse primer	10 picomols/µl(1µl)
DNA	1.5µl
Distill water	16.5 µl
Final volume	25µl

Table 5: The optimum condition of detection GSTM1 and GSTT1 genes

No.	Phase	Tm (°C)	Time	No. of cycle
1-	**Initial Denaturation**	95°C	3 min.	35cycle
2-	**Denaturation -2**	95°C	45sec	
3-	**Annealing**	62°C	45sec	
4-	**Extension-1**	72°C	45sec	
5-	**Extension -2**	72°C	7min.	

4.Determination of DNA damage:

Comet assay using specific Kit (abcam, UK) were used to determine the DNA damage quantitatively in which described as tail movement (TM).

RESULTS

1. **Detection of Polycyclic hydrocarbons in serum**

PAHs transported by blood and reached many tissues and organs. Therefore, this study was conducted to estimate PAHs levels in blood samples of coal making workers. Results showed higher levels of PAHs in all charcoal making workers when compared to non-exposed subjects, this is probably due to their higher concentration in an indoor and outdoor-air around coal-mine located southeast of Wasit province, results summarized in **table (6).**

Table 6: Concentrations of PAHs in exposed population blood (ng/ml)

PAHs	Conentrations ng/ml	
	Exposed populations M±SE	Control M±SE
Naphthalene (NAP)	156±17.18	4±0.8
Acenaphththylene (ACY)	231±8.02	5±0.6
Acenaphthene (ACE)	574±53.11	10±1.2
Fluorene (FLU)	342±12.10	9±1.3
Phenanthrene (PHE)	379±13.12	0±0
Anthracene (ANT)	393±12.32	11±1.1
Fuoranthene (FLA)	165±15.23	0±0
pyrene (PYR)	171±14.56	0±0
Benzo [a] anthracene (BaA)	120±16.34	7±0.9
Chrysene (CHR)	153±17.11	12±1.4
Benzo [b] fluoranthene (BbF)	295±21.31	5±0.6
Benzo [k] fluoranthene (BkF)	213±7.11	10±1.2

2. GSTM1 and GSTT1 genotyping:

Two hundred charcoal-making workers divided into (hundred and fifty males and fifty females) and hundred unrelated healthy subjects which is divided into (sixty-five males and thirty-five females) were studied for deletion polymorphism of these two genes, **figure (1).** The frequencies of these two genotypes for both groups showed in **(Table 7)**. In controls, the frequency of the GSTM1 and GSTT1 null genotypes was 21% (21/100) and 28% (28/100), respectively. While the frequency that reported in this study of the two null genotypes in the charcoal making workers was found to be significantly higher as follows; 28.5% and 31.5% as compared to the controls 21% and 28%. The risk accompanied the GSTM1 and GSTT1deletion polymorphism, it was found that the null genotypes (p=0.0004) and (p=0.012), respectively is related to an increased susceptibility for DNA damage conferring about 3-fold increased risk of developing the DNA damage.

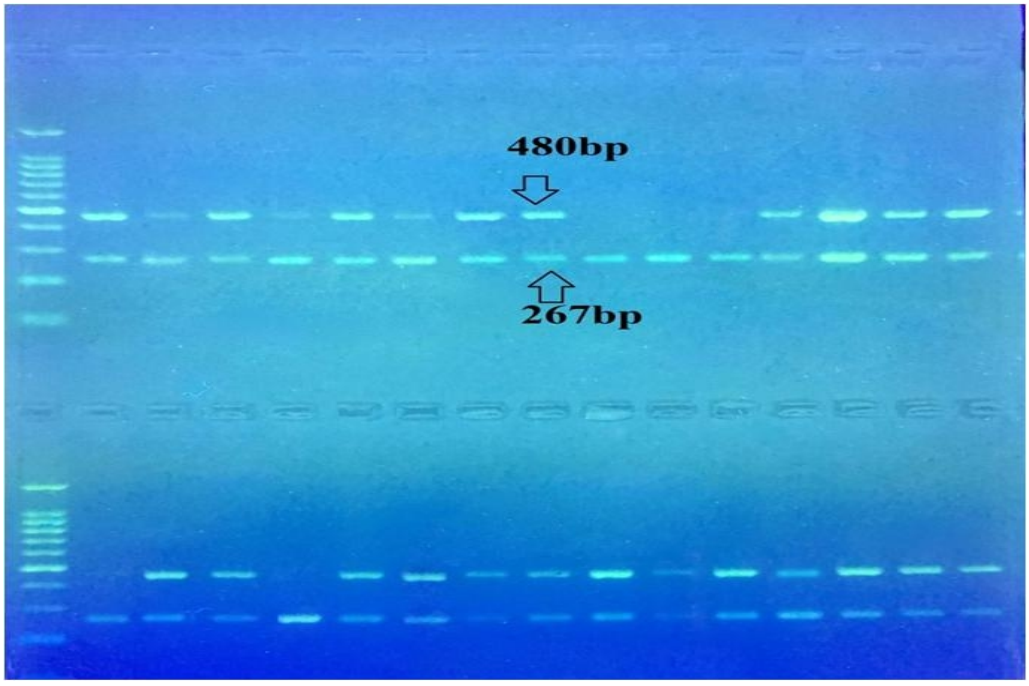

Figure 1: Agarose gel electrophoresis for *GSTT1 + G-GLOBIN* genes (480+267bp). Bands were fractionated by electrophoresis on a 1.5% agarose gel (2 h., 5V/cm, 1X TBE) and visualized under U.V. light after staining with red stain. Lane: 1 (M: 100bp ladder).

Table 7: GSTM1 and GSTT1 genotypes frequencies in charcoal-workers and control.

Gene polymorphism	Exposed (n=200)	Control (n=100)	OR	95% CI	P value
GSTM1					
null	57(28.5%)	21(21%)	4.13	(2.03 – 6.34)	0.0004*
present	143(71.5%)	79(79%)	1		
GSTT1					
null	63(31.5%)	28(28%)	2.89	(2.13 – 8.53)	0.012*
present	147(68.5%)	72(72%)	1		

Confidence interval (95% CI), Odds ratio (OR) and frequency of distribution were calculated.

3. Influences of Gender, age, time of exposure, and GSTs polymorphism on the tail moment (TM):

Current work was studied the influences of sex, age and period of exposure on the damage of DNA in charcoal exposed workers. We noticed that workers who worked for a long time (about 20-40years) were showed significant ($P<0.05$) differences. The results didn't show any significant effect of age, sex except for males were significantly showed an evidence of DNA damage in the charcoal-making workers compared to the non-exposed group. In addition, this work studied the association between the DNA damage and the genetic polymorphism of GSTM1 and GSTT1. The results demonstrated there was an obvious association between the DNA damage and the GSTM1 and GSTT1 null genotypes at ($P<0.05$) in charcoal-making workers **(Table 8).**

Table 8: Influence of Gender, age, exposure, and GST polymorphism on the tail moment.

Factor	Tail moment (µM)			
	Exposed group N(M±SD)		Control group N(M±SD)	
All	200(14.12±0.65)		100(0.31±0.23)	
Gender	Female 50(12.82±0.5)	Male 150(13.12±0.53) *	Female 35(0.32±0.5)	Male 65(0.37±0.33)
Age (years)	25 – 45 158(11.86±0.44)	45 – 65 42(12.24±0.52)	25 – 45 68(0.36±0.41)	45 – 65 32(0.34±0.55)
Exposure (years)	1 – 20 143(11.14±0.54)	20 – 40 57(12.03±0.48) *		
GST genotypes	GSTM1 null 57(12.13±0.52) *	GSTM1 present 143(10.81±0.58)	GSTM1 null 21(0.44±0.36)	GSTM1 present 79(0.31±0.24)
	GSTT1 null 63(12.10±0.55) *	GSTT1 present 137(11.14±0.58)	GSTT1 null 28(0.45±0.39)	GSTT1 present 72(0.4±0.27)

*Significant at $p<0.05$. M= mean, SD= Standard deviation. The comparison of (TM) values were done by using "one-way ANOVA test with post hoc analysis"

4. Detection of DNA damage

The DNA damage was recorded in the charcoal-making workers, the damage showed high significant when compared to controls. The differences between the two groups regarding the DNA damage probably due to the period of occupational exposure and did not depend on the age and sex **figure 2**.

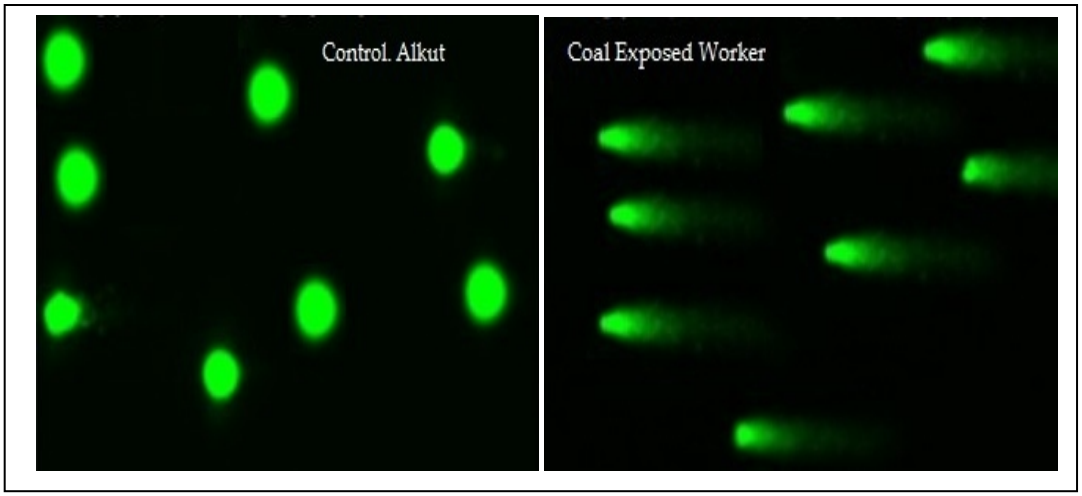

Figure 2: Alkaline comet assay in Exposed workers (A) and Control (B).

DISCUSSION

Many genotoxic studies showed diversity in the levels of genotoxicity indicators in individuals who exposed to high levels of toxic chemicals. Levels of biomarkers that come from the occupational and/or environmental exposure to genotoxicants may be modulated during biotransformation by polymorphic genes with high allele frequency[24]. Information about the actual effect of genetic polymorphisms as biomarkers give an insight into the mechanism of genetic damage that leads to mutagenicity and carcinogenicity[25] and this is, in turn, decreases the risk for exposed populations. It is well acknowledged that GSTM1 responsible for removing PAHs and other toxic metabolites[26]. Therefore, the deletion of the GSTM1 gene may lead to partial or complete loss function of GSTM1[27]. There are numerous studies conducted locally and globally to determine PAHs in different environmental and biological samples. A study conducted in Wasit province (southern to Baghdad) to estimate the Levels of PAHs in the soil samples particularly from different oil fossils sites at "Ahdab oil field- Iraq". The results demonstrated the existing of the PAHs compounds in all soil samples. The total concentrations of the PAHs were ranged from 19mg/kg (site 15) to 855mg/kg (site 16). The main polycyclic aromatic hydrocarbons pollutants in this oil field were found to be Phenanthrene, Fluorene, Pyrene and Fluoranthene [28]. Another study conducted recently by Hassan et al. aimed to detect the fate of the sixteen polycyclic aromatic hydrocarbon compounds (PAHs) as predominant pollutants in the Al-Hussainya River-Karbala Province, Iraq. The results indicated that the concentrations in water samples were (0.24–58.72) ηg. L^{-1}. The concentrations in sediment samples have been observed to be in a range of 0.36–119.06 μg. g^{-1} for naphthalene and benzo (g,h,i) perylene. Benzo(g,h,i)perylene recorded the highest concentrations in different samples of sediment and water as compared with the other compounds[29]. There are limited works studied the concentrations of PAHs in the serum. Pleil et al. measured 22 PAHs in human plasma and found mean cohort values ranging from "0.050 to 0.380ng/ml" (mean= 0.200 ng/ml)[30]. Singh et al. report values of 1.05 to 160.6 ppb (~ng/mL) representing the 25th percentile to 75th percentile values in the distributions of PAH concentrations in the blood of children in Lucknow, India[31]. Kamal et al. measured naphthalene in highly exposed workers in Pakistan. They found naphthalene in 32 of 60 samples with a median value of 155.9 ng/mL. They reported substantial increases (factors of 4 to 6) in naphthalene concentrations in smokers compared to non-smokers for the same occupational classes. Thus, smoking behavior appears to have a substantial influence on blood naphthalene concentrations[32]. Xia et al. were tested the PCDDs/PCDFs and PAHs in the serum samples before and after of the deployment from two hundred persons deployed to Afghanistan or Iraq; in (200) persons not deployed, they found that Naphthalene is the only compound that was measured in a majority of the samples. The other observed species, acenaphthylene (216), acenaphthene (258), fluorene (266), phenanthrene (196), anthracene (240), fluoranthene (234), pyrene (181), benzo(a)pyrene (161), dibenz(a,h)anthracene (145), and benzo(ghi)perylene (170) were found in only 20% to 30% of the samples[33]. PAHs are lipophilic; therefore, it dissolves into lipid membranes of the cells, thus help their absorption through this the skin, gastrointestinal tract and the respiratory tract. Thereafter, the PAHs are spread widely into the body and metabolized to a more soluble substance such as "tetrols, dihydrodiols, dihydrodiol epoxides, epoxides and quinines" after conjugated with glucuronic acid, sulfate or GSH. Covalent binding of PAHs metabolites with DNA to form DNA adducts may lead to mutations and initiation to cancer [34]. Our study revealed a significant ($p<0.05$) association between the level of DNA damage in the charcoal-making workers and GSTT1 and GSTM1 polymorphisms. There are many inconsistent reports about the relationship between the polymorphism and the DNA damage. There are several studies agreed with our results regarding the association between the GSTs polymorphism and DNA lesions in subjects exposed to environmental pollutants such as benzene, styrene, PAHs, and hydroquinone [35-38], respectively. However, there are some studies reported different outcomes who are found no relationship between GSTs polymorphism and DNA damage in charcoal making workers as compared to non-exposed people in a rubber tire factory in Portuguese [38]. In addition, Knudsen et al. observed no marked influence of GSTs polymorphisms on the damage of DNA in oil mine workers in Estonian[40]. In the same way, Marczynski and his colleagues found no significant relationship between GSTs enzymes polymorphisms and genotoxicity in charcoal making workers of graphite-electrode and coke-oven factory[41].

CONCLUSION

Our results showed a relatively high DNA damage in charcoal-making workers, the influence of the genetic polymorphism of the GSTT1 and GSTM1 on the genotoxicity was revealed in a population exposed to PAHs. It has been reported that the GSTs are responsible for detoxification of PAHs and other mutagens and that cells from $GSTM_1$ and $GSTT_1$ null populations were more susceptible to the DNA damage caused by these toxicants. However, more studies with novel study designs are required for a better understanding the interactions between the genes and the environment.

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