Effects of Genetic Polymorphism on Susceptibility to Nephrotoxic Properties of BTEXs Compounds

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Objective: The aim of this study was to ascertain whether genetic polymorphism affects susceptibility of individuals to nephrotoxic potentials of benzene, toluene, ethyl-benzene, and xylenes (BTEXs). **Methods:** Fifty BTEXs exposed workers with one or more abnormal parameter of kidney function and 232 referent subjects, with similar exposure history, free from any abnormal kidney parameters were investigated. Atmospheric concentrations of BTEXs were measured. In addition, genetic polymorphisms were determined by multiplex polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (RFLP). **Results:** The frequencies of GSTP1 Ile-Val/Val-Val, null GSTT1, and null GSTT1/GSTM1 genotypes and mean values of blood urea nitrogen and plasma creatinine were significantly higher, while average glomerular filtration rate was significantly lower in cases than in referent subjects. **Conclusion:** These findings indicate that individuals carrying null GSTT1 or null GSTT1/GSTM1 are more susceptible to nephrotoxic properties of BTEXs compounds.

Keywords: genetic polymorphism, nephrotoxic properties of BTEXs

Petroleum refineries and petrochemical plants are major sources of volatile organic compounds (VOCs) in the environment.[1] A group of VOCs, namely BTEXs (benzene, toluene, ethyl-benzene, and xylenes), are harmful to human health and are classified as hazardous air pollutants (HAPs) by the World Health Organization (WHO).[2] Chronic exposure to BTEXs has been associated with different degrees of neurotoxicity, hepatoxicity, nephrotoxicity, and hematotoxicity.[3] The kidney is vulnerable to toxic chemicals because of the high renal blood flow and its role in transportation, metabolization, and concentration of chemicals present in the tubular fluid.[4] Serum creatinine (CRE) and blood urea nitrogen (BUN) are the primary markers of renal disorders.[5] In addition, estimated glomerular filtration rate (eGFR) is a valuable index of renal function especially in chronic kidney diseases (CKDs).[6,7]

BTEXs are metabolically activated by CYPs enzymes, particularly CYP2 family enzymes.[8] Recently, it has been shown that, under certain conditions, P450s, particularly, CYP2E1 can produce reactive oxygen species (ROS), which results in oxidative stress and cell death.[9] Some studies have shown increased formation of ROS among certain individuals due to genetic polymorphism of *CYP2E1* gene. Increased ROS levels have been considered as causative factors involved in various forms of chronic liver[10] and kidney diseases (CKDs).[11] RsaI/PstI and DraI polymorphisms are known to be the most frequent polymorphisms in CYP2E1. RsaI/PstI polymorphisms of CYP2E1 have been linked with higher transcription. The variant alleles in RsaI/PstI polymorphisms form three genotypes, namely wild−type homozygous (C1C1), heterozygous (C1C2), and variant homozygous (C2C2).[12] The GSTs are phase II enzymes involved in the detoxification of ROS, catalyzing the conjugation reaction between glutathione and compounds containing an electrophilic center, such as chemotherapeutic drugs, carcinogens, environmental pollutants, and other xenobiotics.[13] Therefore, GSTs enzymes play an important role in detoxifying the electrophilic carcinogens.[14] The GSTs family consists of several gene subfamilies. The GSTP1, GSTM1, and GSTT1 are the most important ones.[15] Homozygous deletions of *GSTM1* and *GSTT1* genes result in a complete lack of enzymatic activity, while in GSTP1 genotype, a single nucleotide substitution (A to G) leads to replacement of valine by isoleucine (Ile105val) and decreased enzyme activity.[16]

Some studies have shown that the prevalence and progression of some kidney diseases are significantly different among individuals with different GSTs polymorhism.[17−23] Conversely, there are other reports that do not show any association between GSTs polymorphism and kidney disorders.[24−28] It is of interest to note that most of these studies have been conducted on patients[17−23] and limited studies have examined the role of genetic polymorphism on risk of kidney disorders in workers occupationally exposed to chemical compounds with nephrotoxic potentials.[24−26,28,29]

However, in the light of the above-mentioned controversies as well as a paucity on data related to occupational settings, uncertainty exists regarding a possible similar scenario for kidney disorders. The main purpose of this study was, therefore, to determine whether the genetic polymorphism of *GSTP1*, *GSTM1*, *GSTT1*, and *CYP2E1 RsaI/PstI* genes among employees of a petrochemical plant influences their susceptibility to nephrotoxic potentials of BTEXs compounds.

METHODS

Study Population

This was a cross-sectional study that was undertaken in a petrochemical company in the south of Iran. Male subjects with current occupational exposure to BTEXs for at least 2 consecutive years entered the study. Individuals with diabetes, dyslipidemia, hypertension, cardiovascular diseases, obesity, and a history of exposure to other nephrotoxic chemicals were excluded from the study. In the first phase of this study, biomarkers of kidney function, including CRE and BUN of all employees, were measured. In addition, GFR was estimated using creatinine-based equation.[30] In the second phase of the study, subjects were divided into two groups. The first group included 50 subjects with one or more abnormal parameter of kidney function (cases). BUN and CRE were considered abnormal when they were higher than 25 and 1.6 mg/dL, respectively. GFR was classified abnormal when it was lower than 90 mL/min. The second group included 232 subjects with normal

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kidney function tests (referents). These were selected among 1200 employees of a petrochemical plant. Referent subjects were matched with cases as far as variables such as age, level and length of exposure, job title, and smoking habits were concerned. Furthermore, all subjects were interviewed and a questionnaire pertaining to demographic variables, medical history, smoking habits, alcohol and drug intake as well as occupation-related information (job category, job history, working hours/day, length of employment, occupational or nonoccupational exposure to other chemicals) was completed for them. The study was conducted in accordance with the Helsinki Declaration of 1964 as revised in 2013.[31]

Assessment of Exposure to BTEXs

Sampling and analysis of BTEXs was conducted according to the National Institute for Occupational Safety and Health (NIOSH) method 1501,[32] using activated charcoal sorbent tubes and a personal sampling pump, with a flow rate of 0.1 to 0.2 L/min. Three personal air samples were collected at the breathing zone of each subject during a shift. Likewise, from each administrative office, a sample from the ambient air was collected. The samples were transferred to the laboratory, where they were analyzed by a gas chromatograph equipped with a flame ionization detector (GC-FID) according to NIOSH method 1501.

Genotyping Analyses

Blood samples were taken from all subjects. DNA was extracted from the samples by a commercially available kit (DNeasy blood and tissue kits; Qiagen, Hilden, Germany). DNA samples were stored at −20°C until analysis. Genetic polymorphisms of GSTM1 and GSTT1 were determined by PCR technique as described previously by Singh et al[33] and Cheng et al.[34] Lack of PCR product for GSTM1 or GSTT1 in the presence of the β-globin band was indicative of a null genotype for GSTM1 or GSTT1. Employees with one or two copies of the relevant gene were classified as the ''positive'' genotype and those with homozygous deletions as the ''null'' genotype. Experiments were repeated at least twice according to the standard genotyping protocols. PCR-RFLP technique was used to detect the GSTP1 polymorphisms, as described previously.[34] Homozygous Ile/Ile individuals had a single fragment of 289 bp, and homozygous Val/Val individuals had both 218 and 71 bp fragments. The presence of all three fragments corresponded to heterozygous Ile/Val individuals. Polymorphisms of *CYP2E1* RsaI/PstI gene in the 5-flanking region were identified by PCR-RFLP, using methods previously described by Kamalipour et al[35] and Huang et al.[36] Homozygous c1c1 individuals exhibited a product fragment of 410 bp, whereas homozygous c2c2 individuals revealed a 290-bp and a 120-bp fragment, and heterozygous c1c2 individuals demonstrated all three fragments.

TABLE 1. Demographic Characteristic and Biochemical Parameters of the Studied Groups

Variable (Mean ± SD)	Case ($n = 50$)	Referent ($n = 232$)	P
Age, years	34.01 ± 5.5	34.73 ± 6.78	0.662*
BMI, kg/m^2	25.74 ± 4.06	25.12 ± 4.07	0.159*
Length of exposure, years	7.26 ± 3.9	7.99 ± 4.4	0.949*
Triglyceride, mg/dL	153.85 ± 74.54	145.5 ± 71.41	0.671*
Cholesterol, mg/dL	198.83 ± 35.49	183.96 ± 37.98	0.106*
HDL, mg/dL	45.87 ± 13.79	45.54 ± 10.35	0.858*
LDL, mg/dL(	114.70 ± 26.69	110.04 ± 27.17	0.498*
FBS, mg/dL(	95.57 ± 28.81	92.11 ± 18.20	0.059*
Smoking, no (%)			0.314†
Yes	4 (8)	11 (4.7)	
No	46 (92)	221 (95.3)	
BUN, mg/dL	19.61 ± 4.48	17.85 ± 3.66	0.012‡
CRE, mg/dL	1.29 ± 0.13	1.11 ± 0.15	<0.001‡
GFR, mL/min	81.08 ± 10.42	108.47 ± 15.75	<0.001‡

*Independent-sample t test.
†Chi-square test.
‡Significantly different.

TABLE 2. Mean Arithmetic Concentrations of BTEXs, ppm

Variable (Mean ± SD)	Case ($n = 50$)	Referent ($n = 232$)	P
Benzene	0.31 ± 0.98	0.25 ± 0.79	0.98
Toluene	0.07 ± 0.14	0.11 ± 0.25	0.30
Ethylbenzene	0.23 ± 0.95	0.54 ± 2.43	0.21
O-xylene	0.38 ± 0.83	0.47 ± 1.77	0.61
P-xylene	0.34 ± 0.84	0.42 ± 1.77	0.93
M-xylene	0.34 ± 0.84	0.42 ± 1.77	0.93

Statistical Analyses

Kolmogorov–Smironov test was used to find out whether distribution of the data was normal. Student t test or Mann–Whitney U test was used to compare the means of quantitative variables between the studied groups. Frequencies of categorical variables such as genotype/allele among the studied groups were compared by χ^2 or Fisher exact test. Logistic regression tests were used to determine the association of polymorphism genotypes on the risk of kidney disorders. The association was expressed as an odds ratio (OR) at 95% confidence interval (95% CI). A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Demographic characteristics as well as lipid profile and fasting blood sugar (FBS) of the studied groups are presented in Table 1. No significant differences were noted between both groups as far as demographic variables, exposure history, smoking habits, and biochemical parameters, except kidney function tests, were concerned. The mean values of BUN and serum CRE in cases were significantly higher than those of referent group ($P = 0.012$, $P < 0.001$, respectively). Conversely, the mean value of GFR in cases was significantly lower than that of referent group ($P < 0.001$).

The arithmetic means of BTEXs concentration in the breathing zones of cases and referent subjects are summarized in Table 2. The mean concentration of BTEXs did not significantly differ in the breathing zones of cases and referent subjects.

''Table 3'' depicts the frequencies of GSTP1, GSTT1, GSTM1, GSTM1/GSTT1, and CYP2E1 RsaI/PstI genotypes in both groups. As only few individuals had the GSTP1 Val-Val genotype, those with GSTP1 Ile-Val and Val-Val genotypes were combined. The frequencies of GSTP1 Ile-Val/Val-Val ($P = 0.029$), null GSTT1 ($P = 0.007$), and null GSTM1/GSTT1 ($P = 0.006$) genotypes were significantly higher in cases than in referent subjects. However, no significant differences were noted between two groups as far as the frequencies of GSTM1 and CYP2E1 RsaI/PstI genotypes were concerned.

''Table 4'' exhibits the association between GSTs genotypes and KFT in the studied population. The mean value of serum CRE in subjects with null GSTT1 was significantly higher than its corresponding value for subjects with positive genotype ($P = 0.036$). Conversely, the mean value of GFR in subjects with null GSTT1 was significantly lower than that of individuals with positive GSTT1 genotype ($P = 0.007$). In addition, the mean value of GFR in subjects with GSTP1 Ile-Val and Val-Val genotypes was significantly lower than that of individuals with GSTP1 Ile-Ile genotype

TABLE 3. Frequencies of Genetic Polymorphisms of GSTP1, GSTM1, GSTT1 and CYP2E1 RsaI/PstI in the Studied Groups

Genotypes	Cases ($n = 50$)	Referents ($n = 232$)	*P*
GSTP1			
Ile-Ile	16 (32)	114 (49.1)	0.029
Ile-Val/Val-Val	34 (68)	118 (50.9)	
GSTT1			
Positive	29 (58)	181 (78)	0.007
Null	21 (42)	51 (22)	
GSTM1			
Positive	23 (46)	121 (52.2)	0.263
Null	27 (54)	111 (47.8)	
GSTM1/GSTT1			
GSTM-GSTT1 (+,+)	16 (32)	99 (42.7)	
GSTM1-GSTT1 (+,−) (−,+)*	20 (40)	108 (46.6)	0.006
GSTM1, GSTT1 (−,−)	14 (28)	25 (10.8)	
CYP2E1			
C1C1	50 (100)	225 (97.4)	
C1C2	0 (0)	6 (2.6)	0.595

*+: positive genotype, −: null genotype.

($P = 0.022$). However, there was no statistically significant association between GSTs genotypes and the mean value of BUN.

To control the effects of job title, age, body mass index (BMI), job history, smoking, BTEXs, triglyceride, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and FBS as potential confounders, these parameters entered into the linear regression model. Results showed that, after adjusting for these confounders, there was a significant positive association between GSTT1 and CRE value. In contrast, negative associations were found between GSTT1 and GFR values (Table 5).

Logistic regression model was used to analyze the association between GSTs genotypes and risk of kidney disorders. Table 6 illustrates the results of this analysis. As shown, subjects with GSTP Ile-Val and Val-Val genotypes had a significantly higher risk for kidney disorders, than those with GSTP1 Ile-Ile genotype (OR 2.053, 95% CI 1.074 to 3.92). In addition, subjects with null GSTT1 genotype had a significantly higher risk for kidney disorders than those with positive GSTT1 genotype (OR 2.57, 95% CI 1.353 to 4.883). Similarly, subjects with both null GSTT1 and GSTM1 had a significantly higher risk for kidney disorders as compared with subjects with positive GSTT1 and GSTM1 genotypes (OR 3.46, 95% CI 1.495 to 8.033).

DISCUSSION

Exposure to a mixture of BTEXs has been shown to be associated with oxidative stress as a result of ROS production. ROS, in turn, may cause progression of numerous pathological conditions in the process of metabolism.[37] Oxidative stress has been involved in a number of pathologies, such as neurodegenerative diseases, liver,[38] and kidney[11] injuries. BTEXs and other hydrocarbons are metabolized in the liver by CYP2E1 oxidative pathways, which contribute to the production of ROS.[39] The GSTs are phase II enzymes involved in the detoxification of ROS and catalyze the conjugation reaction between glutathione and compounds containing an electrophilic center, such as environmental pollutants and other xenobiotics.[13] Kidneys are known to be the site of bioactivation of many xenobiotics. Therefore, it would be plausible to assume that in the inactive forms of GSTs, detoxification of the products of oxidative metabolism is disrupted and reduced. This in turn may lead to the damage of renal parenchyma and progression of kidney disorders.[11]

Given the above, we hypothesized that polymorphism of *GSTP1*, *GSTM1*, *GSTT1*, and *CYP2E1 RsaI/PstI* genes, which are involved in biotransformation and detoxification of xenobiotics, may affect susceptibility of individuals exposed to BTEXs compounds. This study was, therefore, undertaken to test this hypothesis.

In the present study, the frequencies of null GSTT1 and GSTM1 genotypes were 25% and 49%, respectively (data not shown), which are similar to those found in a previous study conducted on seven different Iranian populations.[40] Similarly, the frequencies of C1C1 and C1C2 genotypes of PstI/RsaI polymorphisms were 97.9 and 2.1% (data not shown). This observation is in full agreement with the findings of others.[35]

In this study, the frequencies of high-risk genotypes, GSTP1Ile-Val and Val-Val, null GSTT1, and both null GSTM1/GSTT1 genotypes in cases were significantly higher than that of referent group. Limited studies have examined the association between the GSTs genotypes and kidney disorders. Our results are in line with those of previous studies, where the prevalence and progression of kidney diseases have been shown to be linked with some GSTs polymorphism.[17,18,20–23] For instance, Sweeney et al[17] in a case–control study on 130 patients with renal cancer and 505 controls found that the frequency of GSTT1 null genotype but not the GSTM1 and GSTP1 genotypes was significantly higher in patients than in the control group. In addition, Agrawal et al[18] in a study on 184 patients with end-stage renal disease (ESRD) and 569 age- and sex-matched controls from north India found significant associations between null alleles of the GSTM1 and GSTT1 and Val-Val allele of the *GSTP1* gene, with ESRD. Similarly, in the study of Nomani et al[22] on 136 ESRD patients and 137 gender and age-matched healthy controls, the authors showed that the frequencies of GSTM1-null and GST-null genotypes were significantly higher in patients with ESRD, than those in referent group.

In this study, mean values of BUN and plasma CRE were significantly higher, but average GFR was significantly lower in cases than in referent subjects. Similarly, the mean value of CRE in

TABLE 4. The Association Between GSTs Genotypes and Kidney Functions Tests

	GSTP1v		GSTT1		GSTM1	
	Ile-Ile	Ile-Val +Val− Val	Positive	null	Positive	null
	$n = 130$	$n = 152$	$n = 210$	$n = 72$	$n = 144$	$n = 138$
CRE	1.14 ± 0.163	1.15 ± 0.165	1.13 ± 0.16	1.18 ± 0.16*	1.15 ± 0.163	1.15 ± 0.166
BUN	18.12 ± 4.15	18.19 ± 3.62	18.00 ± 3.71	18.61 ± 4.29	18.09 ± 3.83	18.23 ± 3.9
GFR	105.74 ± 17.99	101.80 ± 18.31*	104.90 ± 18.2	99.89 ± 17.98*	103.42 ± 13.63	103.83 ± 22.10

BUN, blood urea nitrogen; CRE, serum creatinine; GFR, glomerular filtration rate.
*Significantly different (independent-sample *t* test, $P < 0.05$).

TABLE 5. Association Between GSTs Genotypes and Kidney Function Tests

	GSTP1			GSTT1			GSTM1		
Dependent Variable	**Beta**	**SE**	***P***	**Beta**	**SE**	***P***	**Beta**	**SE**	***P***
CRE	0.006	0.021	0.789	0.047	0.023	0.04*	0.005	0.02	0.79
BUN	0.188	0.474	0.692	0.577	0.533	0.280	0.186	0.469	0.69
GFR	1.865	2.14	0.385	−5.4	2.39	0.025*	0.603	2.123	0.77

BUN, blood urea nitrogen; CRE, serum creatinine; GFR, glomerular filtration rate.
*Linear regression models, $P < 0.05$ statistically significant.

all subjects (cases and referents) with null GSTT1 genotype was significantly higher than that of positive GSTT1 genotype. Conversely, the mean value of GFR in subjects with null GSTT1 genotype was significantly lower than that of positive GSTT1 genotype. Therefore, it is implied that GSTT1 genotype plays a part in susceptibility of subjects to nephrotoxic properties of xenobiotics.

Our results showed that the risk of kidney disorders in subjects with GSTP1 Ile-Val/Val-Val genotypes significantly increased by 2.053-fold compared with subjects with GSTP1 Ile-Ile (OR 2.053, 95% CI 1.074 to 3.92). However, after adjusting for potential confounders, the risk for GSTP1 genotype was not significant (OR_{adj} 1.69, 95% CI 0.83 to 3.44). In addition, the risk of kidney disorders in subjects with null GSTT1 genotype increased by 2.57-fold compared with those with positive GSTT1 genotype (OR 2.57, 95% CI 1.353 to 4.883). The combination of two high-risk genotypes, null GSTT1 and GSTM1, had the most pronounced effect, in that the risk of kidney disorders increased by 3.46-fold (OR 3.46, 95% CI 1.495 to 8.033). Adjusting the potential confounders resulted in a 4.38-fold increase in the risk of kidney disorders (OR_{adj} 4.38, 95% CI 1.71 to 11.19). Our results are in accord with those of other studies,[17–19,22,23] where an increased risk of kidney disorders have been reported among patients with high-risk genotypes, null GSTT1, and GSTM1 or GSTP1 Val-Val. In contrast, Brüning et al,[24] Buzio et al,[25] Karami et al,[26] and Moore et al[28] reported that high-risk genotypes decreased the risk of kidney disorders in workers occupationally exposed to nephrotoxic compounds.

In the study of Brüning et al[24] on solvent-exposed workers in Germany, GSTT1 null genotype was associated with reduced risk of renal cell cancer (RCC). In addition, in the study by Buzio et al,[25] the presence of GSTT1 genotype increased the risk of RCC among subjects exposed to solvents and pesticides, compared with individuals with GSTT1 null genotype. Similarly, Karami et al[26] in a study on pesticide-exposed subjects showed a significantly elevated risk for RCC in individuals with positive GSTT1 and GSTM1 genotypes compared with unexposed subjects with GSTT1 null genotype. Similar associations reported by Moore et al[28] in a case–control study in central Europe on 1097 cases with kidney cancer, exposed to trichloroethylene, and 10,467 referent subjects.

The reasons for these discrepancies and inconsistencies are not known. However, the explanation of these authors[25,26,28] for the increased risk of kidney disorders among subjects with active forms of GSTs genotypes was the formation of more reactive intermediate from the glutathione conjugation of pesticides and halogenated compounds, mediated by an active GSTs enzyme, which leads to the damage of kidney tissues, while in inactive forms of GSTs (null GSTT1/GSTM1), these reactive intermediates are not formed in the kidney. Although this hypothesis has not been verified by other investigators,[41] glutathione conjugation of aromatic hydrocarbons such as benzene, toluene, xylenes, trimethyl-benzenes, and diethenyl-benzenes, results in the formation of mercapturic acids,[42] which are readily excreted in urine.

Apart from case–control studies, a few meta-analyses have attempted to uncover the relationship between the GSTs polymorphisms and kidney disorders.[27,43,44] The results have not been consistent. da Silva et al[27] and Yang et al[44] in two meta-analysis did not find significant associations between GSTs polymorphisms and RCC risk. Conversely, Cheng et al[43] in a meta-analysis showed that the null genotype of GSTM1/GSTT1 is associated with the risk of RCC in Caucasians and Asians, and there was an association between the dual null genotype of GSTM1/ GSTT1 and the risk of RCC in Asians.

The present study did not find any significant association between polymorphism of CYP2E1 C1C1 and C1C2 genotypes and susceptibility of kidney to toxic effects of BTEXs. This could be explained, at least in part, by the highly polymorphic nature of *CYP2E1* gene resulting in a wide range of enzyme activity levels

TABLE 6. Risk of Kidney Disorders in the Studied Groups

Genotypes	**B**	**SE**	**OR (95% CI)**	**OR_{adj} (95% CI)**
GSTP Ile-Val-Val-Val	0.719	0.330	2.053 (1.074–3.92)	1.69 (0.83–3.44)
GSTP Ile-Ile*			1	1
GSTT1 null	0.944	0.327	2.57 (1.353–4.883)	3.089 (1.5–6.35)
GSTT positive*			1	1
GSTM1 null	0.247	0.313	1.28 (0.693–2.362)	1.46 (0.73–2.93)
GSTM1positive*			1	1
GSTM1-GSTT1 (−,−)	1.243	0.429	3.46 (1.495–8.033)	4.38 (1.71–11.19)
GSTM1-GSTT1 (+,−)(−,+)	0.136	0.363	1.14 (0.562–2.335)	1.36 (0.60–3.09)
GSTM1, GSTT1* (+,+)				

95% CI, 95% confidence interval; OR, odds ratio.
*Referent group, Logistic regression model. $P < 0.05$ considered statistically significant; OR adjusted for job title, age, BMI, job history, smoking; BTEXs, triglyceride, cholesterol, HDL, LDL, and FBS.

among individuals and significant inter-individual variabilities in human CYP2E1 activity.[45,46]

CONCLUSION

In this study, case and control subjects were employees of a petrochemical plant who had similar demographic characteristics, exposure history, and smoking habits. Therefore, increased serum CRE and decreased GFR values in subjects with null GSTT1 as well as increased risk of kidney disorders associated with null GSTT1 and null GSTT1/GSTM1 genotypes, among cases, suggest that the genetic polymorphism plays an important role and is a significant determinant factor in susceptibility to nephrotoxic properties of xenobiotics. Therefore, we suggest that susceptible individuals be screened at their pre-employment examinations to avoid developing serious kidney dysfunction in the future. However, further studies with larger sample sizes, and employees with more clinical and para-clinical findings from occupational exposure to BTEXs mixtures are required to further confirm and substantiate these preliminary observations.

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