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Article

The impact of Occupational Exposure to Petroleum Products on P21 gene Polymorphism and Antioxidant activity among Workers in Thiqar Oil Refinery

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ABSTRACT
The current study was conducted on the Thi-Qar oil refinery workers to assess the effect of polycyclic aromatic hydrocarbons on some biomarkers, including antioxidant activity and *p21* gene polymorphism. A total of 90 samples were further categorized into three groups: the first group was workers exposed directly, the second group was indirectly exposed, and the third group was a control. Blood samples were collected daily and subjected to molecular and antioxidant activity analysis. A significant increase in the concentration of benzene and its byproducts in urine was found in the workers ($P \leq 0.05$) compared with the control. Compared to the control, a statistically significant increase in the percentages of antioxidants among workers directly and indirectly exposed (P=0.001). The frequency ratio of genotypic (CA vs CC) of p21 polymorphism of exposed indirectly and control was 43.3% and 26.6% receptively with a high significant difference (0.002), and odd ratio which amounted to (OR = 5.77) and the genotype frequency ration (AA vs. CC) for the directly exposed and the control group was 40% and 23.3% receptively with high significant difference 0.001, with an estimated odd (OR = 6.0). There is also a high content of hydrocarbons in directly exposed workers' urea. Therefore, more studies still need to be confirmed by investigating a larger sample.
Keywords: Petroleum, antioxidants, *P21* gene, ARMS-PCR

INTRODUCTION

Many countries are dominated by the petroleum sector, which has provided foreign exchange earnings during its modern history and has been used for different reasons by human beings in the manufacturing and petrochemical industries. Petroleum can be used for cooking and lighting fuels at home in industries and for therapeutic reasons. In general, petroleum has been informed to have a toxic effect on human health, causing different diseases and diverse forms

of genotoxic, mutagenic, immunotoxic, carcinogenic and neurotoxic manifestations [1]. Polycyclic aromatic hydrocarbon is considered toxic to the blood and blood-forming organs. Many studies have reported that exposure to these products, in particular for those who work directly in petroleum industries, resulting in reduced lymphocyte, neutrophil and platelet counts in peripheral blood [2]. p21 is one of the factors that promotes cell cycle arrest in response to a variety of stimuli and recently recognized gene. It encodes a nuclear protein of 21 ku, which represses cyclin-dependent kinase action. p21 protein has been reported to work as a basic downstream effectors of p53 and a potential inhibitor of cyclin-dependent kinases. In this way, p21WAF1gene is thought to play a central part in tumor suppression. Changes in p21 expression have been observed in a wide variety of human carcinomas by immunohistochemistry [3].

Long-term exposure to petroleum products leads to continuous production of reactive oxygen species (ROS), which damages DNA, RNA, proteins and other cellular and molecular damage via chemical reactions such as oxidation, nitration, and halogenations, resulting in genetic modification and alterations in the functions of important enzymes and proteins [4,5]. Thus, this exposure may lead to a decrease in antioxidant enzyme activity and hematologic disorders. Several Studies have shown a positive relationship between exposure to PAHs and an increase in the level of reactive oxygen species (ROS) and lipid peroxidation (LPO) as one of the important indicators of oxidative stress [6,7,8].

The present study investigated the adverse effects of petroleum product exposure on the antioxidant activity and p21 gene polymorphism in Thiqar oil refinery workers.

MATERIALS AND METHODS

Ethics statement: This study was approved by the Thi-Qar oil refinery ethics committee. Furthermore, informed approval was obtained from all individuals after thoroughly explaining the project. This form includes age, working hours, working period (years of service), smoking and worker health.

Sample Collection :

The current study was conducted in the city of Thi-Qar, Southern Iraq. It collected 90 samples and divided them into three groups: 30 samples were directly exposed, 30 samples were collected from indirectly exposed, and 30 were healthy control. Five ml of blood was withdrawn and immediately transferred to anticoagulant tubes containing EDTA for molecular study. While Biochemical analysis. Blood was centrifuged at 1500×g for 10 min to collect the serum and store it at -20C° until use for determining antioxidant activity.

P21 rs1801270 Genotyping :

According to the company's instructions, DNA was extracted from blood samples using a gSYNC™ DNA Extraction kit manufactured by (Geneaid, Taiwan). The concentration of DNA was measured by spectrophotometer (Nanodrop, THEERMO, USA), which measures the concentration of DNA (ng/microliter) and verified its purity by reading the absorbance at (260/280 nm).

Two allele-specific (internal) primers are designed in opposing directions and, in combination with common, can simultaneously amplify both the mutant and wild-type alleles. In single-tube PCR, PCR amplification DNA samples were isolated in a 2% agarose gel. Next, genotyping was performed by ARMS PCR. This study used the ARMS technique to determine the presence of the C – A

point mutation with two primer pairs (Table 1). A proliferation fragment of 241bp should be seen in all samples as an indicator of the accuracy of PCR. The expected results of ARMS-PCR in this study included the normal genotype (CC) and the CA and AA mutant genotypes. After PCR, the products were separated on an agarose gel by electrophoresis, and then bands were observed using ultraviolet visualization (Figure 1). The PCR program for amplification was one cycle 5 minutes at 95°C followed by 35 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C, followed by a cycle 5 min cycle at 72°C.

Primer	Sequence (5'-3')	Product size
***P21 (*rs1801270*)* C allele Reverse Wild type Primer**	**ATTAGCGCATCACAGTCGCAG**	**241 bp**
***P21 (*rs1801270*)* An allele Reverse Mutant type Primer**	**ATTAGCGCATCACAGTCGCAT**	
***P21* Common forward Primer**	**GAAGGAGTGAGAGAGACCCT**	

Table 1. The primer sequences used in p21 analyses by ARMS-PCR

Determination of Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay was used to measure antioxidant activity. The absorbance was measured against a blank at 517 nm using a spectrophotometer. Samples were prepared and measured in triplicates. The percentage of scavenging activity of each sample on DPPH radical was calculated as % inhibition of DPPH (%) using the following equation:

DPPH scavenging effect (%)/% Inhibition=A0-A1/A0 × 100

Polycyclic aromatic Hydrocarbons level in Urea: The level of PAH in urea was measured using a chromatography-mass spectrometer - GS Gas based on the method [9]. 500 µL of urine was transferred into 2 ml tube containing chlorobenzene as a standard solution. The solution was injected into The device, and the results were analyzed using a gas spectrometer.

Statistical Analysis

Statistical analysis was evaluated using SPSS software (version 25.0). The data was presented as a mean ± SD and analyzed by one-way ANOVA and Chi-square tests. $P<0.05$ was considered statistically significant. The frequency of the genotypes and alleles within and between groups were shown on the frequency distribution table and were checked for consistency with Hardy-Weinberg equilibrium. The odds ratio (OR) was calculated with a 95% confidence interval (CI).

RESULTS

Demographic characteristics

The mean age of directly exposed workers was 41.12 ± 10.85 years old, 40.13 ± 7.13 years old for indirectly exposed subjects, and that of control subjects was 38.75 ± 10.42 years old. There was no significant difference between exposed

and control subjects in mean age (P = 0.628). The frequency distribution of direct exposed workers, indirect exposed subjects and control subjects according to age was also shown. There was no significant difference in the frequency distribution of study groups according to age (P = 0.128).

Characteristic	Directly exposed *n* =30	Indirectly exposed *n* = 30	control *n* = 30	*P*
Age (years)				
Mean ±SD	41.12 ± 10.85	40.13 ± 7.13	38.75 ± 10.42	**0.628 † NS**
Range	24 – 59	27 – 55	21- 59	
< 30, *n* (%)	9 (30.0 %)	4 (13.3 %)	6 (20.0 %)	**0.128 ¥ NS**
30-39, *n* (%)	2(6.7 %)	8 (26.7 %)	9 (30.0 %)	
40-49, *n* (%)	13 (43.3 %)	16 (53.3 %)	10 (33.3 %)	
≥ 50, *n* (%)	6 (20.0 %)	2 (6.7 %)	5 (16.7%)	

Table 2. Distribution of study groups according to age n: number of cases; SD: standard deviation; †: one way ANOVA; ¥: Chi-square test; NS: not significant at P > 0.05

Regarding smoking, the directly exposed workers' group included 13 (43.3%) cases of smoking and 17 (56.7%) cases of non-smoking. Indirectly exposed subjects included only 2 (6.7 %) cases of smoking and 28 (93.3 %) cases of non-smoking. In comparison, the control group included 3 (10.0 %) cases of smoking and 27 (90.0 %) cases of nonsmoking, and there was a highly significant difference in the frequency distribution of study groups according to smoking (P = 0.001).

Characteristic	Directly exposed *n* =30	Indirectly exposed *n* = 30	Control *n* = 30	*P*
Smoking				
Yes, *n* (%)	13 (43.3 %)	2 (6.7 %)	3 (10.0 %)	**0.001 ¥ HS**
No, *n* (%)	17 (56.7 %)	28 (93.3 %)	27 (90.0 %)	

Table 3. Distribution of study groups according to smoking habit n: number of cases; ¥: Chi-square test; HS: Highly significant at P ≤ 0.001

Detection of P21 rs 1801270 Polymorphism

The polymorphism of the p21 rs1801270 gene was investigated by the ARMS-PCR technique. There are three genotypes (CC, AA, CA) where the homozygous dominant allele (C) was observed at the 241bp locus. The genotype was also seen. Recessive allele (A) at locus 241bp while the heterozygous allele (CA) genotype showed at locus 241bp respectively in the illustrated Figure (1). The

results showed no difference in the genetic distribution from the Hardy-Weinberg equation in all study groups.

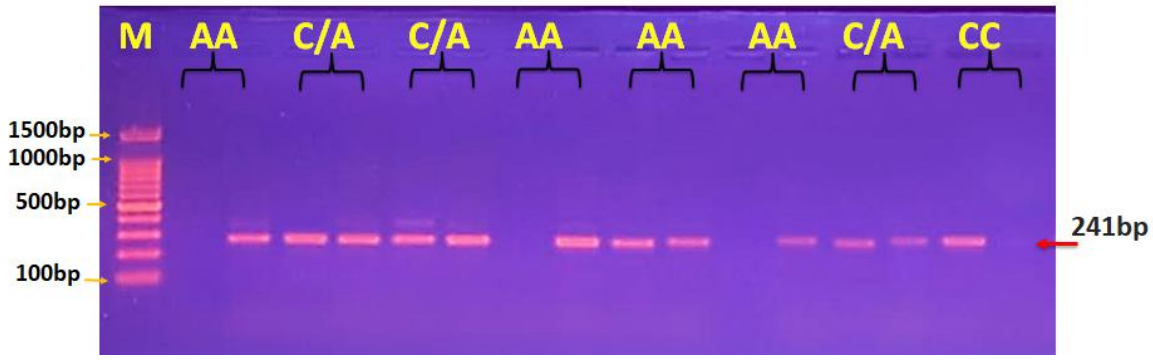

Figure 1. Agarose gel electrophoresis image that showed the ARMS-PCR product analysis of P21 *P21* rs1801270 (C/A) gene polymorphism. Where M: marker (1500-100bp). The lane (CC) wild-type homozygotes were shown as C allele only. The lane (AA) mutant type homozygotes were shown as A allele only, whereas the (C/A) heterozygotes were shown as both C and A allele. The C or A allele presence was observed at 241bp product size.

Figure (1) shows the electrophoresis of the products of the p21 gene. The bundles of this gene are shown after being amplified by the ARMS-PCR technique and transferred to an agarose gel at a concentration of 2% at 100 volts for one hour, where the first column represents the standard evidence (1500-100bp) while, (CC) represents wild-type homozygote, (AA) mutant type homozygote and genotype (C/A) of heterozygote. They are located on the 241bp bundle, respectively.

Frequency of genotypes and alleles of the p21 gene of study groups

The frequency ratio of genotypic (CA vs CC) of p21 polymorphism of exposed indirectly and control was 43.3% and 26.6% receptively with a high significant difference (0.002), and odd ratio which amounted to (OR = 5.77) and the genotype frequency ration (AA vs. CC) for the directly exposed and the control group was 40% and 23.3% receptively with high significant difference 0.001, with an estimated odds (OR = 6.0). The results of the current study showed the frequency of genotypes for the p21 gene in the study groups and the ratio of genotypes (CC vs AA) and (CC vs CA) had a high significant effect at the probability level $P \leq 0.01$, and also the ratio of the frequency of alleles (C and A) had a significant effect at the probability level $P \leq 0.01$ where this study agreed with [13] who indicated in his study the substitution of a C → A polymorphism in codon 31 of exon 2 of the p21 gene and the frequency of the homozygous (Serine / Serine) type where the variant was homozygous (Arginine / Arginine). The frequency of the heterozygous allele (Serine / Arginine) was observed, where a difference was detected at the frequency of the homozygous and heterozygous genotypes. Another study conducted by researcher [14] showed that the genotype (Arg / Arg) is significantly related to an increased risk of colorectal cancer, and a polymorphism in p21 (Ser/Arg) was significantly associated with an increased risk of gastrointestinal tumors.

		Cases–control comparison			
		Directly exposed workers ***n*** **= 30**	**Indirectly exposed workers** ***n*** **= 30**	**Control n = 30**	***P1***
P21 rs1801270 **poly**					
CC		**5**	**6**	**15**	**0.026**
CA		**13**	**12**	**8**	
AA		**12**	**12**	**7**	
Statistic					
		P2	**OR**	**95%CI**	
CA versus CC	**E vs N**	0.761	1.3	0.23 -7.31	
	E vs C	0.002	5.77	1.57-21.14	
	N vs C	0.028	4.44	0.90 -21.87	
AA versus CC	**E vs N**	0.836	1.2	0.211-6.8	
	E vs C	0.001	6.0	1.59 -22.61	
	N vs C	0.020	5.0	0.999 -25.02	

Table 5. shows the genotype of P21 rs 1801270 in the study groups. n : number of cases ; SD: standard deviation; E : workers directly exposed to petroleum products ; N : indirectly exposed workers ; C: control; ¥ : test; Chi-square OR : odds ratio ; CI: confidence interval; HS: highly significant at probability level P ≤ 0.01; NS: It has no significant effect at P > 0.05.

	Cases–control comparison			
	Directly exposed ***n*** **= 60**	**Indirectly exposed** ***n*** **= 60**	***control*** ***n*** **= 60**	***P1***
P21 rs 1801270 **allele**				
A	37	36	22	**0.001**
C	23	24	38	
Statistic				
	P2	**OR**	**95%CI**	
E vs N	0.878	1.07	0.437 -2.62	
E vs C	0.001	2.77	1.32-5.91	
N vs C	0.010	2.59	1.12 -5.41	

Table 6. shows the frequency allele of *P21 rs 1801270* in the study groups. n : number of cases ; SD: standard deviation; E : workers directly exposed to petroleum products ; N : indirectly exposed workers ; C: control; ¥ : test; Chi-square OR : odds ratio ; CI: confidence interval; HS: highly significant at probability level P ≤ 0.01; NS: It has no significant effect at P > 0.05.

Antioxidant activity assessment in the study groups

The study proved that the percentages of antioxidants increased in directly and indirectly exposed persons compared with the control, with a statistically significant difference (P = 0.001).

	Cases–control comparison			
	Directly exposed workers n =30	**Indirectly exposed workers n = 30**	***control* 30*n* =**	***Total P value***
Antioxidant				
Mean	0.204	0.043	0.016	**0.001 † HS**
Range	0.10 – 0.44	0.01 – 0.15	0.01 – 0.05	
FORMULA	DPPH scavenging effect (%)/% Inhibition= DPPH-MEAN/DDPH *100%			
DPPH Value	0.8			
Antioxidant	78.06%	54.44%	74.63%	

Table 7. Comparison of the percentage of antioxidant activity between the direct and indirect exposures and the control group. n : number of cases; SD: standard deviation

PAHs Measurement in the urine of study groups

The results of the current study showed that the level of concentration of hydrocarbons in the urine of people directly and indirectly exposed to petroleum products was higher than the control with statistically significant differences at the probability level ($p \leq 0.05$), as presented in Table 8.

Compounds	**Mean Concentration**		
	Directly exposed workers	**Indirect exposed workers**	**Control**
Type of hydrocarbon compounds			
Benz[a]anthracene	30.291	30.291	0
Chrysene	30.398	30.401	0
Benzo[b]fluoranthene	33.287	33.281	33.314
Benzo[k]fluoranthene	33.347	33.341	33.379
Benzo[e]py...	34.084	34.081	34.093
Indeno[1,2,3-cd]pyrene	36.706	36.695	0
Dibenz	36.778	36.774	0
Benzo[ghi]perylene	37.255	37.255	0
Pyrene	0	0	26.746
P value	0.001	0.001	0.001

Table 8. shows the average concentration of hydrocarbons in urine samples of study groups. *SD: standard deviation; P: ANOVA test; NS: It has no significant effect at $p \leq 0.05$ probability level.*

DISCUSSION

Some studies have shown that codon 31 can be (Ser/Se) homozygote of the p21 gene and is a risk factor for the development of cervical cancer associated with high-risk HPV [10]. The same result was found in the case of esophageal cancer, which showed that the presentation of p21 Ser codon homozygosity confers risks for the development of esophageal cancer [11]. In addition, Benign prostatic hyperplasia (BPH) compared with those with Ser / Ser genotype [12] as this study agreed with the results of the previous studies.

Moreover, the results of this study agreed with [30], who indicated that the high percentage of antioxidants due to the presence of the group hydroxyl group and the unsaturated bonds in the chemical composition of these compounds show a high ability to remove free radicals and prevent oxidation processes and this might be due to the properties of these compounds in curbing free radicals.

Furthermore, the results of this study agreed with those who indicated in his study that continuous exposure to polycyclic aromatic hydrocarbons (PAHs) leads to health risks, and this may be due to the amount of hydrocarbons they were exposed to and for longer periods compared to healthy subjects. The study by [17] to measure the percentage of PAHs in urine by liquid chromatography with a high concentration of six urinary biomarkers are naphthalene, acenaphthene, fluorene, pyrene phenanthrene, and benzo(a)pyrene significantly, which is a biomarker of exposure to PAHs. Carcinogenic confirmed the presence of a cumulative increase of hydrocarbons for exposed workers during successive working days.

CONCLUSION

To conclude, the current study presents PAH-induced toxicities in occupationally exposed workers via the phenomenon of polymorphism of the p21 gene and oxidative stress in the test group compared with the control; this was very clear through an increase in the percentage of hydrocarbons in exposed workers' urea, which is may be an indicator of the occurrence of many diseases in the future. The adverse health effects of hydrocarbon exposure warrant further studies, particularly signaling pathways mutations, which might provide information that prevents disease for various workers working in these places.

Conflict of interest

The authors declared that no potential conflict of interest related to this article was reported.

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