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Research Article

Investigation of susceptible gas station workers to neurological disorders based on evidence of catechol -O-methyltransferase (COMT) gene expression alters

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Abstract

The most dangerous compounds of gasoline are carcinogenic aromatics like benzene and toluene. Aromatic compounds in gasoline can be a mimic neurotransmitter function with the aromatic ring, such as a catechol ring and it interferes in the role of neurotransmitters. Workers who are working in gasoline stations have the highest potential for being exposure to gasoline. COMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to one of the two hydroxyl groups of catecholic compounds, including L-dopa, catechol estrogens, endogenous and exogenous catecholamines as well as their hydroxylated metabolites. This study examine the changes in catechol O-methyl transferees gene expression in people working at the gas station in the city of the Tehran with regard to the determination of harmful gasoline vapors on health and the existence of Aromatic compounds in gasoline. It has been selected 30 workers who have worked at least 5 years in the crowded gas station. Total RNA were extracted from peripheral blood white blood cells of 30 healthy volunteers and 30 workers and then the cDNA was synthesized. This process was followed by Real-time PCR using primer pairs specific for catechol-o methyl transfrase mRNA and beta-actin as internal control. As a result the relative expression catechol-o-methyl transfrase has shown a significant increase (p=0.001) in PBMC of gas station attendants. The results indicate that overexpression of catechol -O -methyltransferase in the target group. Neurotransmitters such as dopamine, norepinephrine, epinephrine and other catecholamines neurotransmitter are catabolized by catechol -O –methyltransferase. Dopamine is associated in mental disorder such as anxiety, depression, sleep disorder, attention deficit /hyperactivity syndrome, restless legs syndrome and Parkinson. It can be concluded that people who work at gas stations is likely higher suffer from such disorders than normal situation.

Introduction

Gasoline is a mixture of over 200 petroleum-derived chemicals and few synthetic products that are added to improve fuel performance [1-4]. Benzene and many more compounds than just the better known BTEX (toluene, xylenes, and ethyl benzene) are the most important hazardous components of the gasoline. These aromatics are transmitted to humans by smell easily. As Benzene and BTEX included in Group carcinogenic substance by WHO, they are very harmful and carcinogenic compounds [5-6]. The workers who had been working at gas station and industries that depend on gasoline exposed to the dangers of aromatic compounds liberated from gasoline fuel. When people fill their car with gasoline is the most common way they are exposed to benzene and other volatile aromatic hydrocarbons. Benzene vapors are also present in exhaust from industries and automobiles. People who work in gas station and live near highways or industries or petroleum station can be exposed to aromatic hydrocarbons. Benzene, toluene, ethyl benzene and xylene also has neurotoxic effect [7]. Aromatic compounds in gasoline can mimic neurotransmitter function with the aromatic ring, such as catechol ring. Dopamine, epinephrine and norepinephrine are Catecholamine neurotransmitters. COMT plays a key role in the metabolism of dopamine and catechol estrogens [8,9]. COMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to one of the two hydroxyl groups of catecholic compounds, including norepinephrine ,epinephrine L-dopa, catechol estrogens, endogenous and exogenous catecholamines as well as their hydroxylated metabolites [9]. The single COMT gene codes for two separate enzymes, soluble (S-COMT) and membrane-bound (MB-COMT) forms [10,11]. The two isoforms of COMT are proposed to have at least partially distinct roles: MB-COMT is believed to be primarily involved in the termination of dopaminergic and noradrenergic synaptic neurotransmission when there are physiologically relevant low concentrations of catecholamine [12]. S-COMT is thought to be mainly responsible for the elimination of biologically active or toxic, particularly exogenous, catechols acting as an enzymatic detoxifying barrier between the blood and other tissues [13-15].

The relation between COMT val158met Polymorphism and incidence of different diseases such as neural Pain, schizophrenia, obsessive-compulsive disorder and breast cancer has been reported in research studies [16-19]. However, over expression and excessive activity of COMT can lead to depression and it can be harmful [9]. COMT is an enzyme that is found in abundance in all tissues in the

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central nervous system. These enzymes are highly expressed in the liver , followed by the kidneys and gastrointestinal tract (both stomach and intestine) [20-21], also it is found in spleen and sub maxillary glands, [22,23] heart, uterus [24], chromaffin cells of the adrenal gland [25], prostate [26], muscle and Human erythrocytes contain some COMT activity [27], blood vessels in dental pulp [28]. Dopamine is synthesized from the amino acid tyrosine and it is precursor of the norepinephrine and epinephrine neurotransmitters [29]. Dopaminergic pathways play an important role in the brain, gastrointestinal system, immune system, pancreas and arteries. Disruptions in dopaminergic pathways in the brain that cause diseases such as, anxiety, violence, depression, Parkinson's disease [30], restless leg syndrome and hyperactivity [31]. Since gasoline are constituents aromatic rings they can act as a substrate of the catechol- o -methyltransferase enzyme therefore they can mimic the role of catecholamine neurotransmitters. According to previous studies it has been hypothesized that the expression of COMT gene has an important role on Individuals who had been working at gas station.

Material and methods

Study population

In this study, subjects divided in two groups, the control group and those working at gas stations. The control group included 30 volunteers resident of the suburban area which they were away from gas stations, with no history of neurological and respiratory disease, smoking, drug addiction and any other special conditions. Actually the members of this group were completely at healthy conditions (Males, aged 20 to 40 years old). The target group was 30 gas station attendants; they worked for at least 6 months, with no history of any mental illness, any psychiatric drugs consumption and allergic diseases. (Males, age range 20 to 40 years old). The participants who smoked or with addiction, and respiratory disease were excluded from this study. Sampling was performed at 3 different gas stations in different areas of the Tehran with the permission of the national oil company. All subjects consented to take part in this study and approved by ethical committee.

PBMCs isolation and RNA extraction

Peripheral blood samples (5 ml) were obtained from the cubical vein and were collected in cell preparation tubes containing an anticoagulant (Heparin). First, blood samples were centrifuged in 300g for 5 min and plasma of samples was preserved for enzyme assay (as substrate). Then, blood samples were diluted with an equal volume of phosphate buffered saline (PBS). Peripheral blood mononuclear cells (PBMC) were isolated from 4 ml of each blood sample by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density centrifugation. Cell density and osmolity were 1.077 ± 0.001 g/ml (20˚C) and 290 ± 15 moms, respectively. Horizontal swing-out centrifuge was used for cell isolation in 850 g for 20 minutes and 1.0 speed regulation. The Buffy coat (lymphocyte layer) was collected and centrifuged in 300 g for 10 minutes and 2.0 Speed regulations. Finally, the resulting pellet was washed in PBS [32].

RT-PCR technique

The total mRNA was isolated from PBMC by High pure RNA isolation Kit (Roche, Germany), according to the manufacturer's instructions. Concentration of extracted RNA samples read with Nanodrop to synchronize all other samples. The RNA (1µg) from each sample was used to synthesize first-strand cDNA by cDNA synthesis kit (Fermentase, Germany). Similarly, cDNA synthesis was carried out based on manufacture's protocols. There were primers designed using oligo5 software (WWW.oligo.net) for COMT and b-actin genes as housekeeping gene based on GenBank sequences and their specificity theoretically checked by BLAST database search against nucleotide reference NCBI database (Table 1).

To confirm the presence of COMT gene in PBMC cells, a common PCR technique was carried out for all samples in a final volume of 20 µl with 1 Unit of Taq DNA polymerase (Sinagene, Iran). Reactionm mixtures contained 2–2.5 mM MgCl, 0.5 mM each of the dNTPs, 0.8–1pM primers, 2.5 µL Taq DNA polymerase (Sinagene, Iran), and 1 µLof the cDNA was used as a template in each RT-PCR reaction. In order to amplify the COMT and b-actin genes, PCR was initiated at 95°C for 5 min and amplified during 35 cycles at 95°C for 1 min, 54 and 62°C for 40 s and 72°C for 1 min and followed by a final extension step at 72°C for 10 min. Finally, the PCR products were visualized by gel electrophoresis on a 2% agarose gel. Moreover, positive control amplification was used experimentally to verify of primers [33].

Real time PCR

Real time PCR was also carried out by a Cyber green flourgenic nucleotide to monitor cDNA amplification by (Roche kit, Germany) measuring the increase in Fluorescence intensity and using primer pairs specific for COMT mRNA and β-actin as the internal control in a Real Time-PCR instrument (Corbett, Germany). The PCR was performed in 10 µL of solution, consisting of 2 µL of Fast Start Master solution and 0.3 µM of each primer. A total of 9 µL of this reaction mix was placed into 0.1 vials, and 1 µL of cDNA was added as a template. Thermal cycling consisted of an initial denaturation step 95°C for 10 min followed by an amplification program (primer annealing, amplification and quantification) repeated for 45 cycles. The amplification program was 95°C for 10 sec, 54 and 62°C for 10 sec, respectively for COMT to β-actin and 72°C for10 sec with a single fluorescence acquisition at the end of the elongation step. The third segment consisted of a melting curve program performed by default program of the real time-PCR instrument. Melting curve analysis showed only one peak for each reaction and this was also confirmed by electrophoresis of PCR products that showed only one band of the expected size [33].

Statistical analysis

First, we used LinReg PCR software for obtaining the Cycle of thereshold (Ct) and efficiency of each reaction, and then data were imported to REST 2009 software, this software calculating is based on pfaffle equation which is as follow: [32]

$$R = \frac{\left(E_{target}\right)\Delta Ct_{target}\left(Mean\ normal - Mean\ sample\right)}{\left(E_{ref}\right)\Delta Ct_{ref}\left(Mean\ normal - Mean\ sample\right)}$$

R: Relative expression ratio of a target gene in comparison to control

E: Real-Time-PCR efficiencies

ΔCP: Crossing point (CP) difference (Δ) of one unknown sample

Table 1. Primer sequences used in RT-PCR and Real time –PCR

Gene	Length PCR (bp)	Primer sequences	Tm(˚C)
B-actin	161	F-5'-AGACGCAGGATGGCATGGG -3'	62
		R-5'-GAGACCTTCAACACCCCAGCC -3'	68
	202	F-5'-CTGGAGGCCATTGACACCTA -3′	64
COMT		R-5′-GGTTGATCTCGATGGTGATGAG -3′	58

Sequencing

COMT and β-actin fragments were sequenced by DNA sequencer ABI 3700 capillary system (Applied Bio System, USA) to confirm amplified sequences [33].

Results

In this study, expression of the COMT gene was evaluated in PBMC of gas station attendants and control groups PBMC (Figure 1).

There was a considerable difference in gene expression rate between gas station attendants and control groups, COMT enzyme gene in PBMC of gas station attendants cases that showed a significant over expression compared to their counterparts in control groups. Furthermore, all sequenced fragments were checked by BLAST database against the nucleotide reference NCBI database and confirmed amplicon sequences.

Expression analysis

Statistical analysis described a significant correlation between COMT specific activity and over expression of the enzyme. The expression of Comt gene in gas station worker PBMCs were significantly higher than in normal people (Table 2).

P value P (H1): the possibility that the increase in the COMTgene expression in comparison to normal people working at the gas station is only due to chance. REF: Reference gene, TRG: Target gene

Discussion

Based on considered hypothesis, the gene expression and COMT activity in PBMCs of gas station attendants was different from healthy individual. Various studies show the relationships between pollutants and diseases such as neurotoxic disease such as autism [34]. Energy consumption has been increased by population growth, industrial development, urban population growth and promotion of social welfare in the world. As a result, the demand for fossil fuels, especially gasoline is increasing every day. Gasoline consumption produces many pollutants. These pollutants and their consequences have detrimental effects on the environment. As noted earlier gasoline has high volatility and gasoline vapors are two categories vapors before and after combustion. Previous studies have focused on post-combustion gasoline vapors, this study investigated the effects of gasoline vapors before combustion .so, this study is step towards improving the health of the workforce at gas stations because most people who are exposed to these vapors are station staff of fuel [35,36]. Moreover, our recent studies show that the higher the gene expression, the greater the comet protein activity. The constant breathing of gasoline has deleterious effects on the nerves system such as imbalance, vibration, acute or sub-acute encephalopathy syndrome [37] jaw jerk, postural tremor, ataxia, abnormal gait, deep tendon reflexes, and affected speech [38]. Another study has shown gasoline station workers reported complaints of headaches, fatigue, sleep problems, memory loss, and general weakness [39]. It is difficult to determine the contribution of benzene exposure to the reported symptoms. Because the benzene content of the gasoline was high; values ranged from 10-17%. Gasoline contain a mixture of c_4 -c_{12} aliphatic, aromatic hydrocarbons, naphthalene, paraffin and alkenes [40-42,37-39]. So, it can be absorb easily through skin and transfer to liver. In liver it converted to triethyl lead. This compound is neurotoxic and it has half time more than 500 days in brain [43]. The aromatic hydrocarbons in petrol are benzene, toluene, xylene and n-hexane. They are lipophilic and absorbed rapidly [40, 42]. The half-life of benzene and toluene in body range from 9-24 h and 7.5h respectively [44-46] Thus, the action of volatile hydrocarbon caused the acute effects of petrol sniffing [45]. Aromatic compound has been known to cause nausea, ataxia and loss of consciousness [44, 47].

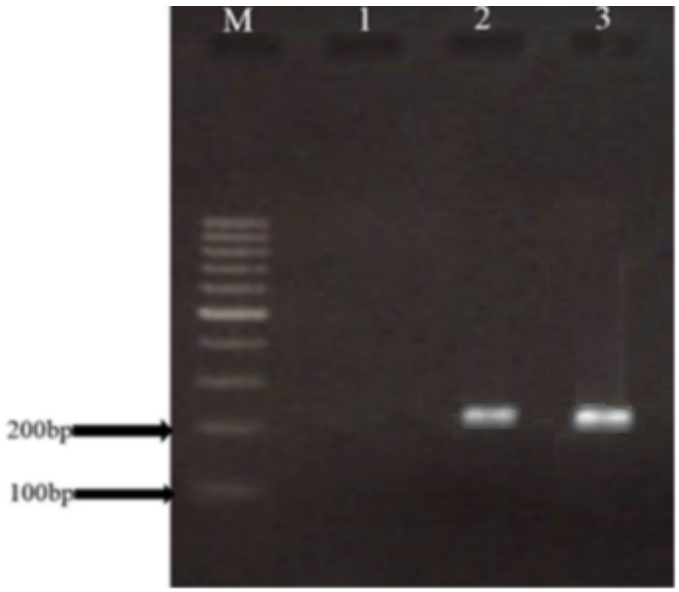

Figure 1. Show COMT gene expression in PBMCs cells. Lane M: Molecular size marker 100bp (Fermentase, Germany), Lane1: control negative, Lane2: control positive of COMT-202bp, Lane3: COMT-202bp

Table 2. Statistical analysis information

Gene	Type	Reaction Efficiency	Expression	Std. Error	P(H1)	Result
B-actin	REF	0.9014	1			
COMT	TRG	0.8531	4.287	3.108 - 141.436	0.001	UP

The composition of gasoline, benzene, toluene and xylene compounds accounted for a significant percentage of gasoline. The dangers of breathing exhaust and vapors of gasoline and releasing gasoline vapors during fueling is more dangers than contact with the skin. In the study by Amal A Kinay was observed that the Rats exposed to unleaded gasoline were decreased norepinephrine levels in the cerebral cortex and hypothalamus. Also in this study was observed norepinephrine levels declined in the hippocampus of mice that were exposed to leaded gasoline and unlead [48]. Gasoline also are reduces the level of dopamine in the hypothalamus and the hippocampus. The MMT leads to fluctuations in dopamine levels. MMT is an organic manganese, which is added in unleaded gasoline [48]. Manganese has antioxidant properties and reacts with the dopaminergic system [49].

Also proved that Mn reacts with dopamine, which it can activate intracellular ROS and NOS signaling pathway, as a result induced dopaminergic cell death. J. Mann in 1999 proved that level of monoamine neurotransmitter such as norepinephrine and dopamine be reduced in depression people [50]. Also it have been showed that gasoline and in mice causing anxiety and emotional problems [48].

Benzene as a main compound in the gasoline is involved in Synthesis and catabolism of catecholamines and serotonin in the brain, causing neurotoxic effects. Many study have shown attention deficit hyperactivity disorder (ADHD) related to the reduction of activity (amount) of dopamine. The disease, restless legs syndrome (RLS) related to the reduction of activity (amount) of dopamine [31]. The search for

effective treatment of MDD (major depressive disorder) and other depressive disorders has developed the physiological role of dopamine in depression [48]. evidence from clinical trials suggests that in patients with depression, the major metabolite of cerebrospinal dopamine (Homovanillic Acid (HVA) are reduced in the central nervous system. There is some evidence that dysfunction of dopaminergic neurons in Parkinson's disease may also lead to motor symptoms, depression and cognitive disorders [29].

The Catecholamine-O-methyltransferase (COMT) enzymes in the synaptic cleft reduce catecholamines such as dopamine, epinephrine and epinephrine. The most important effect of this enzyme is clearing of dopamine in different regions of brain. So, it is involved in neurological disease that dopamine decrease create them such as major depression and Parkinson [9, 49, 50].

Conclusion

In this study, catecholamines (COMT) gene expression was risen in people who were exposed to gasoline fumes. Based on lower levels of catecholamine neurotransmitters associated with the vapors of gasoline, it can be concluded that increased expression of this gene leads to increase decomposition of catecholamines such as dopamine and norepinephrine in people who are exposed to these vapors. As a result, these people are more prone to neurological disorders such as major depression, anxiety, Parkinson's and other diseases that that is relevant with reduced dopamine and other catecholamine neurotransmitters.

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