

Assessment of Changes in Expression of the 5-HT_{2A} and GABA_A Receptor Genes in Peripheral Blood Mononuclear Cells of Gas Station Workers, Tehran, Iran

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Abstract

Gasoline containing different chemical compounds especially aromatic compounds such as benzene, xylene, toluene and ethylbenzene which are the most dangerous compounds. Gas station workers have the highest potential for exposure to gasoline evaporation. Many studies have investigated the effect of gasoline vapors and cause of death in people working in gas stations. According to reports, the cause of death of these people is mainly due to cardiovascular disease, lung cancer, leukemia and other cancers in the world. With regard to the harmful effects of gasoline vapors on health and the importance of the relationship between the immune system and nervous, in this study we investigated alternations in gene expression pattern of the serotonin 5-Hydroxytryptamine 2A (5-HT_{2A}) receptor and type A gamma-aminobutyric acid (GABA_A) receptor in peripheral blood mononuclear cells (PBMCs) of people working in gas stations, Tehran. For this purpose, The PBMCs were separated from whole blood of 30 gas station workers and 30 normal by Ficoll-hypaque. The total RNA was extracted and the cDNA was synthesized. This process was followed by Real-time PCR using specific primers for 5-HT_{2A} and GABA_A receptor mRNAs. Results have shown a significant increase ($P = 0.001$) in relative gene expression of 5-HT_{2A} receptor whereas relative expression of GABA_A receptor was significantly decreased ($P = 0.004$) in PBMC compared with normal control peoples. According to the role of the serotonin in asthma and due to the importance of the activity of this neurotransmitter through 5HT_{2A} receptor which has a significant increase in patients with allergic asthma it can be concluded that workers in the gas station may be more susceptible to developing allergic asthma. On the other hand, low expression of GABA_A receptor is associated with mental health problems such as depression, so it can also be concluded that people working at the gas station are more exposed to these health problems in comparison to normal people.

Keywords: Gasoline, Gas Station, Gene Expression, 5-HT_{2A} Receptor, GABA_A receptor, Real-time

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Introduction

At a gas station during delivery, storage, and dispensing the fuel to customers, unburned fuel can be released to the environment in either liquid or vapor form. Fuel is a complex mixture of chemicals especially aromatic compounds such as benzene, xylene, toluene and ethylbenzene which are the most dangerous agents with toxic and carcinogenic effects. For instance, occupational studies have linked benzene exposures to numerous cancers, including acute myeloid leukemia and acute non-lymphocytic leukemia. Based on scientific reports, concerns have been raised that gasoline vapor exposure at the gas station by attendants and also tanker truck drivers may result in health risks [1, 2]. Accordingly, evaporation of the fuel and its organic matters can be caused physiological damage, including damage to the nervous system, eye irritation, nose and throat, genetic mutation, the birth of premature infants, and so on. Also, harmful environmental effects, including acid rain production, photochemical smell phenomena, global climate change, damage to the ozone layer, rise in global

warming, and so on [3, 4]. A study in Canada showed that in 2009 the amount of evaporative losses at gas stations was 58,300,000 L. With a population of about 34 million, they estimated that about 1.7 L of gasoline was released per capita per year in Canada from evaporative losses, without counting the liquid spills [1] which, of course, seems to be more widespread in developing countries. Since gasoline vapor condensation is higher at gas stations so worker in these places are people with greatest exposure to organic compounds originating from gasoline which increases the risk of health for them [2]. These people spend most of their time in these places, approximately 40 h per week for years, where vapors from the pump are at their highest concentrations, with benzene concentrations measuring between 30 and 230 ppb in the breathing zone [5, 6]. Generally, different quantitative toxicity metrics exist for gasoline inhalation. According to the EPA Integrated Risk Information System (IRIS) [7], 0.03 mg/m³ (about 9.4 ppb) is considered as a reference concentration, corresponding to decreased lymphocyte

counts, whereas based on the National Institute for Occupational Safety and Health (NIOSH) standards, recommended exposure limit (REL) is a time-weighted average concentration (for up to a 10-hour workday during a 40-hour workweek) of 0.319 mg/m³ or about 100 ppb [8] which represent high risks for health. Based on these topics, in recent years many studies have been done on harmful effects of gasoline but most of these studies focused on petrol vapors after combustion. In this study, we investigated the effects of gasoline vapors prior to combustion on workers at the gas station by assessment of alterations in gene expression pattern of the 5-HT_{2A} and type A γ -aminobutyric acid receptor genes in PBMCs which can be used as molecular markers in order to identify potential health risks. Between the immune system, the nervous system and the endocrine is a close connection by cytokines, secretion factors from immune cells, hormones and neurotransmitters. Serotonin is one of the keys and most important neurotransmitters that has been shown to have different receptors on different tissues of the body as a peripheral hormone and can thus control the immune system by the nervous system. Most of the body's serotonin is circulating in the bloodstream, transported by blood platelets that during it by binding to immune cell surface receptors like 5-HT_{2A}, can lead to a change in their function [9]. In recent years, a number of immunoregulatory functions have been described for serotonin. For the instant, serotonin modulates cytokine secretion in monocytes/macrophages or can also suppress the release of tumor necrosis factor- α (TNF- α) and interleukin-1 β by activating serotonin receptors. Furthermore, neutrophil recruitment and T-cell activation can both be mediated by serotonin [10]. On the other hand, it is proven that serotonin 5-HT_{2A} receptor function as a contributing factor to both neuropsychiatric cardiovascular and disorders. Studies have been showed that 5-HT_{2A} receptor can cause some neuropsychiatric disorder such as Schizophrenia and Depression/anxiety [11]. Also, γ -aminobutyric acid (GABA) is the most important inhibitory neurotransmitter in the central nervous system (CNS) and plays an important role as a trophic factor to modulate several essential developmental processes including neuronal proliferation, migration, and differentiation. Studies showed that GABA inhibits or reduces the activity of the neurons or nerve cells in CNS and plays an important role in behavior, cognition, and the body's response to stress. Accordingly, lower-than-normal levels of GABA in the brain have been linked to schizophrenia, depression, anxiety, and sleep disorders [12, 13]. Molecular and biochemical studies have led to identification three major GABA receptors, termed GABA_A, GABA_B and GABA_C receptors [14]. Based on biomedical reports, GABA_A receptors are of great clinical significance in several disorders, including epilepsy, anxiety, and alcoholism [15-17]. Therefore, as noted above, the expression level of this receptor can be used as molecular markers to assess the probability of related disorders.

Materials and Methods

Recruitment of subjects

Thirty healthy male volunteers, between 23 and 55 y of age, were randomly selected from among the workers at

the gas station (Tehran, Iran), who even worked for 6 months at the station. Selection was based on some exclusion criteria including tobacco smoking, history of mental illness or taking psychosocial drugs, having asthma and any allergies. Controls were also selected among people who lived in a normal weather zone. Subjects were informed about the design and purpose of the study and provided full informed written consent. Also, this case-control study was approved by National Institute of Genetic Engineering and Biotechnology (NIGEB) Ethics Committee.

Isolation of peripheral blood mononuclear cells (PBMCs) using Ficol-Hypaque

Peripheral blood is the primary source for investigation of the human immune system. A rapid and simple method of purifying PBMCs is Ficoll-Hypaque density gradient centrifugation based on the density differences between mononuclear cells and other elements in the blood sample [18]. For this purpose, the peripheral blood should not be older than 8 h and supplemented with anticoagulants such as EDTA. Accordingly, the venous blood sample was collected from all participants and aliquoted into tubes containing EDTA to separate PBMCs. In the following, PBMCs were isolated from fresh blood using Ficoll-Hypaque (Lympholyte-H; Cedarlane Laboratories, Hornby, ON, Canada) gradients centrifugation. After washing with PBS at 4°C, isolated PBMCs were counted and viability was tested using the Naebaur counting chamber after staining with Trypan blue.

Total mRNA extraction and cDNA synthesis

To analysis mRNA expression, total RNA was extracted from PBMCs using a Total RNA Extraction Miniprep kit (Takara, Japan) according to the manufacturer's instruction. DNA was eliminated during RNA extraction utilizing DNase I. The concentration of RNA was quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific) and RNA purity was determined by the 260/280 nm absorbance ratio, and also RNA integrity was assessed by agarose gel electrophoresis. Finally, the complementary DNA (cDNA) was synthesized using One-Step RT-PCR Kit (Takara, Japan). At first, cDNA was used for qualitative assessment of expression of 5-HT_{2A} and Type A γ -aminobutyric acid receptors with primers which synthesized by Macrogen, Korea (Table 1). An Eppendorf thermocycler (Germany) was used for all reactions.

Table 1. Primer sequences.

Gene		Primer sequences
β -actin	F	5'AGACGCAGGATGGCATGGG -3'
	R	5'-GAGACCTTCAACACCCAGCC -3'
5HT _{2A}	F	5'-CCATCCAGAATCCCATCCACC-3'
	R	5'-GGACAAAGTTATCATCGGCGAG-3'
GABA _A	F	5'-TCAACTACACGGACCACACG-3'
	R	5'-TTGGTGGTTCCGGATCCTTGG-3'

F: Forward; R: Reverse

Quantitative real-time PCR

The qPCR assay was performed by StepOne Plus instrument (Applied Biosystems, USA) for assessment of

changes in expression of 5-HT_{2A} and Type A γ -aminobutyric acid receptors with specific synthesized primers (Table 1). All reactions were carried out in duplicate, and each reaction was contained 20 μ l of mixture solution consisting of 10 μ l of SYBR PCR Master Mix (Applied Biosystems, USA), 1 nM concentration of each primer, 3.5 μ l of sterilized deionized water and 4.5 μ l of cDNA. Also, β -actin was used as a calibrator control to evaluate expression and mRNA level of target genes. The cycling conditions were as follows: one cycle of 95°C for 10 min as the holding time, 95 °C for 45 s for denaturation, and 55°C for 45 s as annealing/extension time. To evaluate of gene expression 40 cycles was set as a cycle cut-off point for no detection. During the annealing/extension time amplification data were collected. Melting curve analysis was performed as follows: reactions were heated to 95°C for 15 s, followed by cooling to 60°C and stepwise heating to 95°C with a ramp rate of 0.3°C. Finally, the comparative threshold cycle (CT) method (using the formula $2^{-\Delta\Delta(CT)}$) was used to analyze the data obtained. A Corbett thermocycler (Australia) was used for all reactions.

Statistical Analysis

The statistical significance of the differences between the control and test values was evaluated using one-way ANOVA t-test using SPSS 19 software (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at a p-value <0.05.

Results

The mRNA level of 5-HT_{2A} and Type A γ -aminobutyric acid receptors in PBMCs of workers and control subjects is shown in Fig. 1 & 2. As depicted in these figures, the difference between the expression of two markers in worker subjects and controls were statistically significant ($P < 0.05$). Accordingly, expression level of the 5-HT_{2A} receptor in worker subjects was significantly ($P = 0.001$) increase compared with controls (Fig. 1). By contrast, relative expression of GABA_A receptor was significantly ($P = 0.004$) decreased in their PBMCs compared with normal control peoples (Fig. 2). Based on our results, 5-HT_{2A} receptor expression in PBMCs isolated from the blood of workers showed a 5-fold increase compared with control people while GABA_A receptor expression in PBMCs decreased about 3-fold in comparison to PBMCs isolated from the blood of normal people.

Discussion

Environmental pollution is one of the problems that humankind faced since the beginning of the industrial revolution to today. Among various types of pollution, air pollution is one of the most important environmental and public health issues especially in large cities [19]. Generally, this type of contamination is the result of fossil fuels, which are escaped in the air before or after combustion. According to many studies, exposure to ambient air pollution has been associated with a series of adverse health effects such as physiological changes in pulmonary functions and the cardiovascular system. Also, more recently, associations have been demonstrated between chronic exposure to air pollution and negative neurological effects

[20]. In recent decades, reasons such an increase in population growth of the world, industrial development, urban population growth and the promotion of social welfare have led to increased energy consumption in the world. As a result, demand for fossil fuels especially gasoline has been increasing every day [21] which can lead to production a large number of chemical particles and vapors in the air with devastating effects on health and environment.

As mentioned above, gasoline has two types of vapors which are produced before combustion, which is related to its volatility, and after combustion. Previous studies focused on gasoline vapors after combustion, but in this study for the first time we examined the effects of vapors from evaporative emissions of gasoline on workers at the gas stations because they are the main group exposed to these vapors. Generally, gasoline emission due to fuel handling and storage at gas stations is as a major problem for people working in these stations. Due to gasoline volatility, many chemical particles as volatile organic compounds (VOCs) are easily released in the gas station environment. According to reports typical gasoline mainly consists of volatile organic hydrocarbon and aromatic compounds such as Benzene, Toluene, Xylene (BTX), C8-, C9-, methanol, 5 MTBE, butenes and pentenes [22].

A study by Eisaei et al. (2015) in Tehran, showed that the average concentrations of BTX and total VOCs at gas stations are 161.22, 200.81, 229 and 647.01 ppm, respectively, higher than the World Health Organisation (WHO) guidelines [23]. VOCs such as BTX are known as predominant pollutants and have adverse effects human health [24, 25]. Studies have been demonstrated that short-term (acute) exposure to high VOC concentrations can cause dizziness, tremors, nausea, or other health problems. Also, long-term (chronic) exposure to some VOCs especially Benzene may cause cancer related to liver, kidney, lung, heart and the brain caused by DNA strand breaks, chromosomal damage, etc [23, 26, 27]. On the other hand, various studies have shown the relationship between these compounds and the occurrence of various diseases, including allergies [28, 29] and neuropsychological symptoms especially by BTX [30]. Accordingly, in a study by Gordian *et al.*, (2010), the relationship between gasoline emissions and Asthma symptoms was investigated [29]. They measured the indoor air Benzene, Toluene, Ethylbenzene and Xylenes (BTEX) in over 500 houses with attached garages in Anchorage, Alaska. Based on their report, BTEX compounds especially Benzene can be effective in developing Asthma. Thetkathuek *et al.*, (2015) investigated neuropsychological effects of the Toluene and Xylene among workers which exposed to them in two paint manufacturing factories. Based on their reports high amounts of these compounds can cause neuropsychological effects, especially psychosomatic symptoms, and sleep disturbances [30]. In molecular level, some studies have been shown that high concentration of blood serotonin is associated with the intensity and exacerbation of asthma disease. Other studies showed that 5-HT_{2A} can enhance T-cell blastogenesis and production of pro-inflammatory cytokines such as IFN γ [31]. On the other hand, some reports discussed the role of 5-HT receptors in airway smooth muscle

contraction. Cazzola and Matera reported that four groups of 5-HT receptors including 5-HT_{1A}, 5-HT_{2A}, 5-HT_{3A}, and 5-HT₇ are important in controlling airway functions and serotonin directly has its contractile effect through 5-HT_{2A} receptor on smooth muscle cells [32-34]. Accordingly, in a study we investigated the expression level of 5-HT_{2A} gene in PBMCs of patients with allergic Asthma [31].

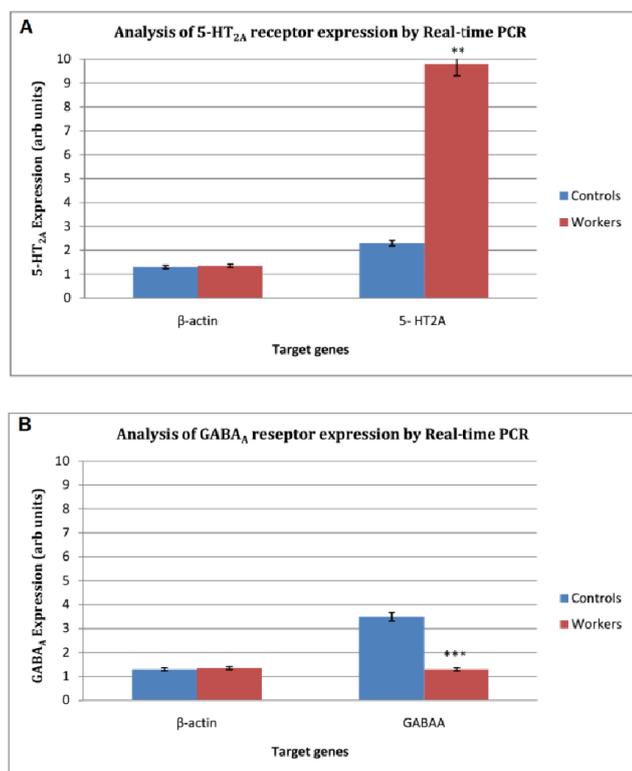


Figure 1. Quantitative analysis of the expression level of 5-HT_{2A} (A), and GABA_A (B) receptor genes in PBMCs which isolated from workers at gas stations and control subjects. qPCR analysis shows that the expression level of 5-HT_{2A} and GABA_A genes significantly increased and decreased, respectively, in comparison with control. Data are presented as mean ± SEM (error bars) from three independent experiments (**P=0.001 & ***P<0.004).

Our results indicated that gene expression is significantly increased in PBMCs of asthma patients in comparison with the normal group. Therefore we suggested that the expression level of 5-HT_{2A} gene in PBMCs could be considered as a biomarker of asthma. As previously mentioned, exposure to air pollutants can lead to lung inflammatory responses such as allergic asthma. Based this topic and our results in the previous study, we analyzed gene expression level of the 5-HT_{2A} receptor in PBMCs of people who had been exposed to air pollution in the highly polluted area compared to allergic asthmatic patients [35]. The expression level of 5-HT_{2A} gene in PBMCs of both exposed individuals and allergic asthmatic patients was significantly increased in comparison with control group. These results indicate that there may be an association between increase expression level of 5-HT_{2A} receptor gene and exposure to air pollution as well as asthma incidence. Along with our studies, Amidfar *et al.*, (2017) investigated expression levels of the 5-HT_{2A} receptor in PBMCs of patients with the

major depressive disorder (MDD) [36]. According to their results, mRNA expression of 5-HT_{2A} receptor was significantly higher in PBMCs of all patients in comparison with healthy controls.

They showed that 5-HTR_{2A} mRNA levels in PBMCs of MDD patients could be associated with the severity of depression and the duration of the illness. Similar to these reports, many studies have shown that GABA_A receptors are also involved in the pathology of several neurological and psychiatric diseases such as epilepsy, anxiety, autism, depression, sleep disorders and Alzheimer's disease [37]. For example, according to investigations, a significant decrease in the expression level of GABA receptors has been determined in the brains of individuals with autism. Related results show that GABA_A receptors are reduced in the brain regions that have been associated with the pathogenesis of autism [38]. Also, studies have demonstrated a relationship between reductions in specific subtypes of the GABA_A receptor and incidence fragile X syndrome [39, 40]. Based these topics, in current study we hypothesized that alterations in gene expression pattern of the 5-HT_{2A} and type A γ -aminobutyric acid receptor genes in PBMCs can be used as diagnostic molecular markers in order to identify potential health risks such as allergic Asthma and neurological and psychiatric diseases especially for attendants at gas stations which are exposed to high level of gasoil and VOCs.

Conclusion

Our results showed that there is a significant relationship between the gasoline vapor level and the change in expression level of 5-HT_{2A} and GABA_A receptor genes. So, gasoline pump workers have a higher risk of developing some diseases such as allergic Asthma and psychiatric diseases compared to normal people but this requires further studies on a larger number of patients.

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