Original Article

The Effects of Exposure to Petrol Vapours on Growth, Haematological Parameters and Oxidative Markers in Sprague-Dawley Male Rats

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Abstract

***Background*: Petrol is known to be hazardous to human health and is associated with various health effects, such as haematotoxicity and oxidative stress. Although Malaysia has adopted the European fuel quality standards in recent years in order to reduce petroleum pollutants and to improve air quality, gasoline with research octane number 95 (RON95), believed to contain benzene and other toxic substances, is still widely used all over the country. This study assessed the effect of RON95 gasoline on haemtological parameters of rats after 11 weeks of exposure.**

***Methods:* A total of 16 male Sprague-Dawley rats were randomly divided into two groups: control (exposed to ambient air daily) and gasoline exposed (exposed to petrol fumes at 11.13 ± 1.1cm3/h for 6h daily, 6 days/week) groups. Body weight was monitored daily. At the end of 11 weeks, the rats were sacrificed, bone marrow was extracted for cytological examination, and blood samples were collected for a full blood picture examination, full blood counts and oxidative markers.**

***Results:* The results show that gasoline inhalation was associated with a significant ($P < 0.05$) reduction in the rate of weight gain and a reduction in mean corpuscular haemoglobin concentration and red cell distribution width. It was also observed that the inhalation of gasoline was associated with changes in the nuclei of megakaryocytes, hence causing an increase in the percentage of abnormal megakaryocytes with detached nuclei, hypo-lobulation and/or disintegration. However, the inhalation of gasoline did not cause significant changes in oxidative markers in the erythrocytes.**

***Conclusion:* This study shows that 11 weeks of inhaling RON95 petrol vapours caused adverse effects on weight gain, blood cell indices and bone marrow megakaryocytes, but did not cause significant changes in oxidative markers in erythrocytes. The definitive effects of these changes on health require further confirmation.**

Keywords: *gasoline, bone marrow, red blood cell indices, haematological parameters*

Introduction

Gasoline, or petrol, is a volatile liquid with a complex mixture of aliphatic and aromatic hydrocarbons. It is commonly used as fuel for internal combustion engines and is also used as a thinner, decorative agent, and industrial solvent. Some of its constituents are known to be highly toxic or carcinogenic to humans (1,2). Many of the toxicological effects associated with the exposure to gasoline can be attributed to specific components of gasoline, such as benzene, toluene, ethylene and xylene, which are also known as volatile organic compounds (VOCs) (3,4). A number of studies have demonstrated that the occurrences of various health problems are closely associated

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with occupational exposures to VOCs (2,5,6). Uzma et al. (6), found that exposure to benzene from petrol vapour caused haematotoxicity among petrol station workers. This study also found significantly diminished pulmonary function that was associated with duration of exposure to gasoline vapour. Furthermore, other studies have also suggested that there is a causal relationship between industrial exposures to benzene and the incidence of some types of leukaemia and aplastic anaemia (7,8). A number of experimental animal models have been used to determine the toxicological effects of gasoline inhalation (9–12).

Recently, there has been increasing concern for environmental safety and health hazards related to gasoline exposure, and as a result, several strategies have been put in place to remove the potentially toxic compounds from the gasoline mixture. For example, the usage of leaded gasoline was substituted by non-leaded fuel. Likewise, some of the potentially noxious components were also eliminated from the petrol blend, and oxygenates were added to enhance engine performance (13,14). These strategies have resulted in the production of reformulated gasoline blends without harmful heavy metal additives and with relatively lesser amounts of noxious constituents such as benzene (15). The Euro II standard recommends that the research octane number (RON), which is a measure of performance of a fuel, should be 97, and the standard recommends a maximum sulphur content of 500 ppm and a maximum volume of benzene of 5% (16). In an attempt to improve the air quality in Malaysia, the Malaysian government proposed to move to the Euro II standard by the end of 2007 (16). Despite that effort, unleaded RON95 petrol that is believed to contain benzene and many other toxic substances is still commonly used in Malaysia. Therefore, the purpose of this study was to determine the effects of unleaded RON95 petrol vapours on the rate of weight gain, haematological parameters and oxidant/antioxidant markers in rats after an 11-week exposure period.

Materials and Methods

Animals

The procedure used in this research was approved by the animal ethics committee of Universiti Sains Malaysia (USM), Malaysia. A total of 16 Sprague-Dawley male rats, with 8 in each group (170–230 g by weight and 6–7 weeks old), were supplied by the Animal Research and Service Centre, USM. The animals were maintained on a standard and balanced rat's diet and were allowed free access to food and water ad-libitum. The animals were allowed to adapt to the room conditions for a minimum of seven days before commencing the experiment. These rats were kept in a standard cage with commercial pine chip bedding in a well-ventilated animal room with a 12h day/night cycle.

The rats were divided into two groups by a simple randomisation method: control and exposed groups. Rats in the control group were exposed to normal air, and those in the exposed group were exposed to gasoline according to a modified technique described by Uboh et al., (17).

Petrol and dosage of exposure

The unleaded RON95 gasoline used in the study was purchased from a local Petronas Petrol filling station. Rats in the exposed group were housed in their cages (two rats per cage) and exposed to gasoline vapour in the exposure chamber, while rats in the control group were kept in a gasoline-vapour-free section of the experimental animal house. During the exposure period, the cages were positioned in a Plexiglas exposure chamber with 100 cm x 90 cm × 150 cm dimensions. The room condition was monitored and maintained at 55 ± 10% relative humidity and temperature at 28 ± 3 °C. Four calibrated 1000mL beakers, each containing 500mL of petrol, were put in the chamber one hour prior to the commencement of the exposure to ensure that the exposure chamber was saturated with gasoline vapour. The exposed group was placed in the exposure chamber and allowed to inhale the evaporating vapours in the chambers during the exposure period of 6 h (9:00 am to 3:00 pm) daily, six days per week. At the end of every exposure, the animals were transferred back to the vapour-free section of the experimental animal house. During the exposure period, the initial and final volumes of liquid gasoline were respectively recorded before and after daily exposure. The daily differences in volume were used to estimate the relative concentrations of vapours used in this exposure method. The average dosage of exposure in this study was 11.13 (SD 1.1) cm^3/hour, 6 h daily, six days per week for 11 weeks.

Determination of weight changes

The weight change and growth rate were carried out as in Uboh et al., (18). Briefly, the body weight was measured daily with a digital balance (Sartorius AG, Germany) throughout the experimental period. Daily body weight was taken in the morning before the exposed group was

taken to the chamber. The body weight changes were expressed as weekly percentage weight gain and percentage growth rate, where the percentage weight increase was calculated from the formula:

$$\frac{(FBW\text{-}IBW) \times 100,}{IBW}$$

and the percentage growth rate was calculated from the formula:

$$\frac{(FBW\text{-}IBW) \times 100,}{Y}$$

where,

FBW = final body weight
IBW = initial body weight
Y = Number of days exposed

Blood sample collection and analysis

Blood samples were collected at the end of the 11 weeks experimental period. The rats were sedated with intraperitoneal (IP) ketamine at 90mg/kg body weight for the collection of blood sample via cardiac puncture. Two millilitres of the blood sample were aliquoted into ethylene-diaminetetraacetic acid (EDTA) tubes for full blood counts (FBC) and differential counts (DC); then the remaining amount was put into heparinised tubes for measurement of oxidant/antioxidant markers. The rats were then sacrificed and dissected for the collection of bone marrow. Bone marrow from the femur was taken instantly, and smears were prepared within 2–3 minutes of death. The method of bone marrow collection was as described by Bolliger (19).

Full blood counts were determined using an automatic blood count analyser. Blood smears were stained with Wright-Giemsa stain and examined under the microscope for blood cell morphology and white blood cell (WBC) differential count. The preparation of the bone marrow smear followed the push smear technique as reported by Bolliger (19). One drop of fetal calf serum was put at one end of a glass slide. The collected bone marrow was gently mixed with the serum by a paintbrush. The material was then spread in an even film by a spreader. Slides were left to air dry. The smear was then stained by May-Grunwald-Giemsa (MMG) staining. Cytologic evaluation of bone marrow was carried out by the determination of erythroid and megakaryocytic cell lines.

For the determination of oxidant/antioxidant markers in erythrocytes, each assay was carried out by using a microplate-based, colorimetric method commercial kit (Cayman, USA). Blood samples in the EDTA tubes were centrifuged at 1000g for 10 minutes at 4 °C. Plasma was separated, and the packed red blood cells (RBCs) were washed three times with 4–5 volumes of cooled saline. The erythrocyte was lysed in four times its volume of ice-cold deionised water; it was then centrifuged at 10 000g for 15 minutes at 4 °C. The supernatant (erythrocyte lysate) was collected and stored in multiple aliquots at -80 °C and thawed only once before analysis. Each assay was performed in duplicates on the same day.

Statistical analysis

Statistical analysis was carried out using SPSS 18.0.1. A normality test was performed to assess the data distribution, and it was observed that only the body weight was normally distributed. A two-way mixed-design ANOVA was used to determine the changes of body weight between groups and over the experimental period. A Mann-Whitney U test was used to determine the significance of the differences between the groups for the other measured parameters. Differences were considered significance at $P < 0.05$. The results were reported either as mean (SD) or median (IQR).

Results

No statistically significant difference in the initial body weight was observed between both groups prior to the commencement of the experiment. The weekly body weight gain following the 11 weeks duration of gasoline inhalation is presented in Figure 1. The time main effect and the experimental group x time interaction effect on weight gain over the 11 weeks exposure period were tested using the multivariate criterion of Wilk's lamda (Λ). The experimental group x time interaction effect was significant: $\Lambda = 0.216$, $F(1,10) = 72.40$, $P < 0.001$. The results showed a trend in which the petrol exposed group of animals had a lesser rate in weight gain compared with the non-gasoline exposed group. This suggests that exposure to petrol vapours significantly alters body weight gain. The results of the total weight gain and percentage of weight gain (PWG) at the end of the 11 weeks experimental period (Table 1) show that the exposed group had a significantly lower PWG compared with the control group ($P < 0.001$).

For the blood analysis, no observable abnormality was seen in the peripheral blood film

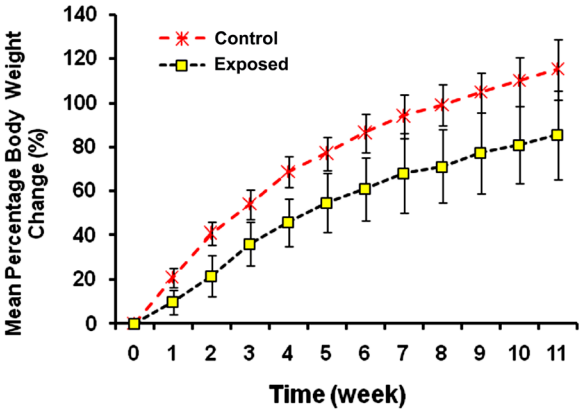

Figure 1: The weekly percentage weight gain of the control and exposed groups. A mixed-design ANOVA was carried out to assess the effect from the inhalation of gasoline fumes on rats' weight gain for the duration of 11 weeks. The time main effect and the experimental group x time interaction effect were significant ($P < 0.001$). The test indicated that there was a significant difference in the growth rate between the control and exposed groups. At the end of the 11 weeks experimental period, the exposed group had a significantly lower PWG compared with the control group.

of the exposed group after the 11 weeks inhalation period. Results of full blood counts (FBC), red blood cell (RBC) indices, and the differentials count of the control and exposed groups are presented in Table 2. They show that mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW)values in the exposed group were significantly lower than the control group values ($P = 0.006$ and 0.026, respectively).

The bone marrow cytology examination showed that both groups had cells at various stages in the hematopoietic process. No obvious abnormality of erythroid lineage was observed in either group. However, the magakaryocte lineage showed a significantly higher percentage of abnormal megakaryocytes in the petrol-exposed rats compared with the control after 11 weeks of the experiment (Table 3). Figure 2a shows a normal mature megakaryocyte from the control group with multiple nuclei but slightly detached from each other, giving rise to a pleormorphic megakaryocyte. Figure 2b shows another normal megakaryocyte with multiple nuclei fused together to form a lobulated mass. Figure 3a and 3b show abnormal mature megakaryocytes (from the petrol-exposed group) with detached and hypo-lobulated nuclei, respectively.

In general, the results of the oxidants/antioxidants makers in the erythrocyte lysates of the control and exposed rats were not significantly different. However, the superoxide dismutase (SOD) and glutathione peroxidase (GR) activity of experimental petrol exposed group (EPG) rats displayed a tendency to be higher than that in the control. The concentration of malondealdehyde (MDA) in erythrocyte showed no statistically significant difference ($P = 0.773$) between the two groups.

Discussion

The present study shows that inhalation of petrol vapour at 11.13 ± 1.1cm^3/h for 6 hours daily, 6 days per week for 11 weeks caused no mortalities in the experimental rats. This is consistent with previous findings in which rats survived between 6 to 13 weeks of exposure to petrol vapour (20–23). The weight of both control and exposed groups of rats was monitored and expressed as weekly percentage weight gain (PWG). An associated reduced weight gain was found in the petrol-exposed group; this is consistent with the report of Uboh et al, (24). This effect is proposed to arise from complex interactions between various gasoline components and a signalling pathway involving intercellular and molecular mechanisms that result in the suppression of growth stimulatory signals and the stimulation of growth stimulatory pathways and that subsequently cause growth retardation and weight loss (12). Exposure to petrol in rats may occasionally be associated with severe and life-threatening weight loss, as encountered in this study.

Haematological analyses, which included full blood counts, RBC indices and bone marrow examination, provided information about the haematopoietic responses of rats to gasoline vapours. The results indicate that the inhalation of gasoline fumes for 11 weeks is associated with changes in some of the haematological parameters, that is, reduction in MCHC and RDW values. The results support earlier reports on the haematoxicity associated with exposure of rats to petrol fumes (12,24). Even though no significant changes were observed in the rest of the haematological parameters, it is imperative to highlight the changes observed, as this will offer valuable information with respect to changes

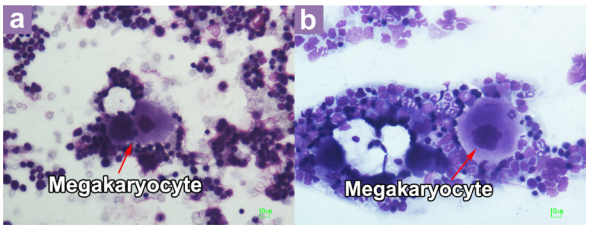

Figure 2: Normal mature megakaryocyte from the control rats. (a) Representative photomicrographs of May-Grunwald-Giemsa (MGG) staining (400× magnification) of bone marrow showing a normal megakaryocyte with slight nuclear detachment (mild pleormorphism) and (b) another one with fused nuclei forming a lobulated mass; both are from the normal control group.

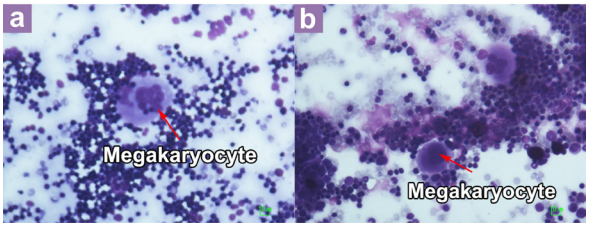

Figure 3: Abnormal mature megakaryocyte from the exposed rats. Representative photomicrographs of May-Grunwald-Giemsa (MGG) staining (400× magnification) of bone marrow showing (a) an abnormal megakaryocyte with detached nuclei and (b) another abnormal megakaryocyte with hypo-lobulated nuclei; both are from the gasoline exposed group.

Table 1: Weight gain of rats following the eleven weeks of experimental period

Group (n = 8)	n	Mean body weight (g) Mean (SD)		PWG in %
		Initial	final	
Control (NCG)	8	204.7 (14.0)	440.4 (42.0)	115.1 (13.5)*
Exposed (EPG)	8	204.0 (10.2)	378.3 (42.3)	85.6 (20.1)

Comparison of percentage body weight change between control and exposed groups of rats. * $P < 0.001$, percentage weight gain = PWG, Data were analysed using independent *t* test.
Abbreviation: NCG = normal control group; EPG = experimental petrol exposed group.

Table 2: FBC, RBC indices, and DC of the control and exposed rats after 11 weeks of experimental period

Parameters	Median (IQR)		*P* values
	Control (n = 8)	Exposed (n = 8)	
RBC (10^{12}/L)	8.1 (0.17)	8.3 (0.50)	0.051
Hb (g/dl)	15.9 (0.25)	15.6 (0.63)	0.328
PCV (%)	48.0 (1.00)	50.0 (3.20)	0.161
MCV (fl)	58.5 (3.50)	59.0 (1.00)	0.574
MCH (pg)	19.0 (1.00)	18.5 (0.50)	0.130
MCHC (g/L)	33.5 (1.50)	31.5 (1.20)*	0.006
RDW (%)	14.1 (0.88)	12.7 (0.85)*	0.026
TWBC (109/L)	8.7 (1.23)	8.7 (2.43)	0.645
Polymorphs (%)	29.0 (6.00)	29.5 (2.20)	0.442
Lymphocytes (%)	67.5 (6.50)	68.5 (13.50)	0.505
Platelets (10^9/L)	750.0 (68.00)	754.0 (37.00)	0.645

Mann-Whitney U test was used to compare the differences of the measured parameters between control group and exposed group.
Abbreviation: RBC = red blood cells; Hb = haemoglobin; PCV = packed cell volume; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; RDW = red cell distribution width; TWBC = total white blood cells.

Table 3: The percentage of abnormal megakaryocytes of both control and exposed groups after 11 weeks of experimental period

Group	Percentage of abnormal megakaryocytes Median (IQR)
Control	2.7 (2.4)
Exposed	16.7 (4.5)*

Comparison between control group and exposure group was by using Mann- Whitney U test.

in the red cell indices. The significance of the reduction in RDW is not clear, but there is some possible implication for the reduction in MCHC. MCHC is derived by dividing the haemoglobin (Hb) by the PCV. Changes in PCV, RBC count, and Hb will affect the MCHC and MCH values. It was particularly observed that the exposed rats tended to have higher RBC counts and PCV values compared with the control rats. The rise in PCV could also be associated with lowered pulmonary function (25). This has been reported by Lykke et al., who demonstrated that rats exposed to gasoline vapour for about seven weeks began to exhibit signs of pulmonary distress and showed a progression of lesions characteristic of fibrosing alveolitis after 12 weeks (25). Hence, the elevated RBC levels and PCV values in the exposed rats found in this study could be unexplained or could be ascribed to reactive processes that lead to the activation of the bone marrow, with consequent increases in these parameters. It may be associated with exposure to a mixture of inhaled air and the constituents of gasoline vapours, causing mild tissue hypoxia and leading to the stimulation of RBC production.

However, the interpretation of these findings should be done with caution, as they may not represent the exact situation in humans. The adverse effect of petrol inhalation on haematological parameters in rats, which is dependent on the amount of various constituents and the duration of exposure, has been previously reported (23). The alterations in haematological indices due to exposure to various components of petrol (such as benzene) are not constant, especially at lower levels (26) such as those found in different samples of petrol. Furthermore, the extent of petrol-exposure-induced injury to tissues is dependent on quantity, timing and the design of exposure (6). The absence of observable changes in the peripheral blood cell morphology in this study may be attributed to several factors: (a) the period of exposure might not have been long enough to cause obvious abnormalities in the peripheral blood cells; (b) the concentration of noxious constituents of the gasoline used could have been minimised to a lower level; (c) a combination of these factors could be responsible; or (d) the gasoline that was used in the study was devoid of a significant amount of harmful substances, such as benzene, hexane and other hydrocarbons. Thus, further studies should be conducted to determine the dose of harmful compounds (such as benzene) that are present in the currently used formulation of Malaysian RON95 gasoline.

From the bone marrow examination, as shown in Figure 3a and 3b, the inhalation of petrol fumes was associated with detachment and/or hypo-lobulation of megakaryocytic nuclei. These cells were counted, and their percentages were calculated with respect to normal magakaryocytes, as shown in figure 2a and 2b. It should be noted that those megakaryocytes with mild nuclear separation were considered normal; this is because of previous findings suggesting that an uncommon minor pleormorphism characterised by the presence of poorly fused nuclei with variable nuclear:cytoplasmic ratios is occasionally observed in rats' bone marrow (19).

In this study, the inhalation of gasoline fumes was observed to be accompanied by a high percentage of abnormal megakaryocytes in the marrow of exposed animals. Although these qualitative changes did not give rise to a significant difference in the peripheral platelets count of the affected group, it was found that the petrol-exposed rats tended to have elevated RBC counts in comparison with the control group. Hence, this may suggest an early affectation of the peripheral RBC count due to adverse changes in the bone marrow. However, this requires further evaluation. Although no obvious alteration was found in other cell lineages and the general myeloid-to-erythroid (ME) ratio of the marrow, it cannot be completely established that the inhalation of petrol fumes is not associated with subtle changes in these cell lineages. The reasonably large size of the megakaryocytes helped considerably in identifying the nuclear abnormalities associated with the inhalation of constituents of petrol in the exposed rat models. It is therefore recommended that future studies should be conducted to delineate the probable subtle alterations that may be associated with petrol exposure in those cell lineages. This could be achieved by extending the exposure duration or using electron microscopic scanning, which

is more sensitive. In addition, epidemiological studies have shown that repeated exposure to high concentrations of benzene causes cytogenetic damage and benzene-associated leukaemia, aplastic anaemia, and pancytopenia (5,7,8,27). Benzene is not a direct-acting agent in the bone marrow, but it is converted to metabolites which cause haematotoxicity. The mechanism of petrol-induced adverse effects on haematological parameters and bone marrow could be described in two ways based on an examination of benzene by Snyder and Hedli (28). Some studies have demonstrated that benzene metabolites are capable of covalent interaction with cellular macromolecules, such as DNA, as well as protein inhibition and RNA synthesis. This could result in bone marrow depression (29). The other possible mechanism could be due to the ability of benzene metabolites to induce oxidative stress with a consequent alteration in the DNA structure (28). Both of these subsequently lead to mutagenesis, and this is believed to deter the normal haematopoietic process. With the consideration that petrol comprises more than 500 hydrocarbons and benzene is one of its major noxious substances, it could be expected that the adverse effects of petrol on haematological indices and bone marrow reported in the present study may arise from similar mechanisms with those reported for benzene.

Exposure to petrol fumes can cause oxidative stress in many ways. For example, benzene, which is a common constituent of gasoline, may undergo metabolic activation to give rise to metabolites, such as hydroquinone and 1, 2, 4-benzenetriol with the consequent generation of ROS, which could lead to an impairment of antioxidant activity and thus, the stimulation of oxidative stress (10,30–33). RBCs' high levels of oxygen and Hb (a strong promoter of oxidative process) render them highly susceptible to oxidative damage (34), and they represent a reliable model for the study of oxidative stress in the laboratory (35). Overall, this study did not show any significant differences in all the oxidative markers examined; however, a fluctuation in these markers was observed, hence suggesting an imbalance in the antioxidant defence mechanism. However, it should be noted that there is a discrepancy as to the activity of some oxidant enzymes and the levels of other oxidative markers in petrol-induced toxicity reports; these depend on the type of tissue examined as well as the study protocols among other factors. While some studies have reported elevated activity, some have reported lowered activity, and others have reported insignificant findings (10,30–33).

Conclusion

The present study indicates that 11 weeks of exposure to Malaysia RON95 gasoline vapours is still associated with some significant alterations in a few haematological parameters and in bone marrow megakaryocytes; however, these effects are milder compared to those in previous reports on leaded gasoline.

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Conflict of Interest

None.

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Authors' Contributions

Conception and design: MBAB, WZA, SAS, BSA
Analysis and interpretation of the data: MBAB, WZA, BSA
Drafting of the article: MBAB, BSA
Critical revision of the article for the important intellectual content: WZA, SAS
Final approval of the article: WZA, SAS, BSA
Statistical expertise, obtaining of funding: BSA
Collection and assembly of data: MBAB

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References

1. Mehlman MA. Dangerous properties of petroleum-refining products: carcinogenicity of motor fuels (gasoline). *Teratog Carcinog Mutagen*. 1990;**10(5)**: 399–408. doi: 10.1002/tcm.1770100505.

2. Caprino L, Togna GI. Potential health effects of gasoline and its constituents: A review of current literature (1990-1997) on toxicological data. *Environ Health Perspect*. 1998;**106(3)**:115–125. doi: 10.1289/ehp.98106115.

3. Kirchstetter TW, Singer BC, Harley RA, Kendall GR, Hesson JM. Impact of California reformulated gasoline on motor vehicle emissions. 2. Volatile organic compound speciation and reactivity. *Environ Scie Technol*. 1999;**33(2)**:329–336. doi: 10.1021/es980374g.

4. Madhoun A, Ahmed W, Ramli NA, Yahaya AS, Yasuf MD, Fitri NF, et al. A Framework For Monitoring And Modelling Of Btex In Various Development Statuses In Penang, Malaysia [Internet]. Reposit USM (MY): ICENV; 2008 [cited 2014 Feb 15]; Available from: http://eprints.usm.my/13180/1/a_framework_for_monitoring.pdf.

5. Rinsky RA, Hornung RW, Silver SR, Tseng CY. Benzene exposure and hematopoietic mortality: A long-term epidemiologic risk assessment. *Am J Ind Med*. 2002;**42(6)**:474–480. doi: 10.1002/ajim.10138.

6. Uzma N, Khaja MS, Kumar BS, Aziz N, David MA, Reddy VD. Impact of organic solvents and environmental pollutants on the physiological function in petrol filling workers. *Intern J Environ Res Pub. Health*. 2008;**5(3)**:139–146. doi: 10.3390/ijerph5030139.

7. Cronkite EP, Drew RT, Inoue T, Hirabayashi Y, Bullis JE. Hematotoxicity and carcinogenicity of inhaled benzene. *Environ Health Perspect*. 1989;**82**:97–108. doi: 10.2307/3430767.

8. Rinsky RA, Smith AB, Hornung R, Fillon TG, Young RJ, Okun AH, et al. Benzene and leukemia. An epidemiologic risk assessment. *N Engl J Med*. 1987;**316(17)**:1044–1050. doi: 10.1289/ehp. 89821 89.

9. Huff JE, Haseman JK, DeMarini DM, Eustis S, Maronpot RR, Peters AC, et al. Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F1 mice. *Environ Health Perspect*. 1989;**82**:125–163. doi: 10.1289/ehp. 8982125.

10. Kinawy AA. Impact of gasoline inhalation on some neurobehavioural characteristics of male rats. *BMC physiol*. 2009;**9**:21. doi: 10.1186/1472-6793-9-21.

11. Li D, Yuan C, Gong Y, Huang Y, Han X. The effects of methyl tert-butyl ether (MTBE) on the male rat reproductive system. *Food Chem Toxicol*. 2008; **46(7)**:2402–2408. doi: 10.1016/j.fct.2008.03.024.

12. Uboh FE, Eteng MU, Ebong PE, Umoh IB. Vitamins A and E reverse gasoline vapors-induced hematotoxicity and weight loss in female rats. *Toxicol Ind Health*. 2010;**26(9)**:559–566. doi: 10.1177/0748233710373080.

13. Ahmed FE. Toxicology and human health effects following exposure to oxygenated or reformulated gasoline. *Toxicol Lett*. 2001;**123(2–3)**:89–113. doi: 10.1016/S0378-4274(01)00375-7.

14. Schuetzle D, Siegl WO, Jensen TE, Dearth MA, Kaiser EW, Gorse R, et al. The relationship between gasoline composition and vehicle hydrocarbon emissions: a review of current studies and future research needs. *Environ Health Perspect*. 1994;**102(Suppl 4)**:S3–S12. doi: 10.1289/ehp.94102s43.

15. Costantini MG. Health effects of oxygenated fuels. *Environ Health Perspect*. 1993;**101(Suppl 6)**:S51–S60. doi: 10.1289/ehp.93101s6151.

16. Tan P. Government sets new Euro II standard for fuel [Internet]. Malaysia (MY): paultan.org; 2007 [cited 2013 Feb 14]. Available from: http://paultan.org/2007/02/15/government-sets-new-euro-ii-standard-for-fuel/

17. Uboh FE, Akpanabiatu MI, Eyong EU, Ebong PE, Eka OO. Evaluation of toxicological implications of inhalation exposure to kerosene fumes and petrol fumes in rats. *Acta Biologica Szegediensis*. 2005; **49(3-4)**:19-22.

18. Uboh FE, Akpanabiatu MI, Atangwho IJ, Ebong PE, Umoh IB. Effect of vitamin A on weight-loss and heamatotoxicity associated with gasoline vapours exposure in wistar rats. *Int J Pharmacol*. 2008;**4(1)**: 40–45.

19. Bolliger AP. Cytologic evaluation of bone marrow in rats: indications, methods, and normal morphology. *Veterin Clinic Pathol*. 2004;**33(2)**:58–67. doi: 10.1111/j.1939-165X.2004.tb00351.x

20. Poon R, Park G, Viau C, Chu IH, Potvin M, Vincent R, et al. Inhalation toxicity of methanol/gasoline in rats: effects of 13-week exposure. *Toxicol Ind Health*. 1998;**14(4)**:501–520. doi: 10.1177/074823379801400402.

21. Uboh FE, Akpanabiatu MI, Ekaidem IS, Ebong PE, Umoh IB. Effect of inhalation exposure to gasoline on sex hormones profile in Wistar albino rats. *Acta Endocrinol-Bucharest*. 2007;**3(1)**:23–30. doi: 10.4183/aeb.2007.23.

22. Uboh FE, Akpanabiatu MI, Eteng MU, Eyong EU. Exposure To Gasoline And Kerosene Vapours: A Risk Factor For Nephrotoxicity In Rats. *Internet J Toxicol*. 2010;**7(2)**.

23. Uboh FE, Akpanabiatu MI, Eteng MU, Ebong PE, Umoh IB. Toxicological effects of exposure to gasoline vapour in male and female rats. *Internet J Toxicol*. 2008;**4(2)**.

24. Uboh FE, Akpanabiatu MI, Alozie Y, Edet EE, Ndem JI, Ebong PE. Comparative Effect of Vitamins A and E on Gasoline Vapours-Induced Haematotoxicity and Weight-Loss in Male Rats. *Intern J Pharmacol*. 2009;**5(3)**:215–221.doi: 10.3923/ijp.2009.215.221.

25. Lykke AWJ, Stewart BW, O'connell PJ, Le Mesurier SM. Pulmonary responses to atmospheric pollutants. I: an ultrastructural study of fibrosing alveolitis evoked by petrol vapour. *Pathol*. 1979;**11(1)**:71–80. doi: 10.3109/00313027909063541.

26. Browning E. Toxicity and metabolism of industrial solvents [Internet]. United Kingdom (UK): CABdirect; 1965 [cited 2013 Feb 13]. Available from: http://www.cabdirect.org/abstracts/19662703238.html;jsessionid=970F5EB737A301D538D03F93FAA724A6.

27. Aksoy M. Hematotoxicity and carcinogenicity of benzene. *Environ Health Perspect*. 1989;**82**:193–197. doi: 10.1289/ehp.8982193

28. Snyder R, Hedli CC. An overview of benzene metabolism. *Environ Health Perspect*. 1996;**104 (Suppl 6)**:1165–1171. doi: 10.1289/ehp.96104s61165.

29. Rushmore T, Snyder R, Kalf G. Covalent binding of benzene and its metabolites to DNA in rabbit bone marrow mitochondria in vitro. *Chem Biol Interact*. 1984;**49(1–2)**:133–154. doi: 10.1016/0009-2797(84)90057-7.

30. Georgieva T, Michailova A, Panev T, Popov T. Possibilities to control the health risk of petrochemical workers. *Int Arch Occup Environ Health*. 2002; **75(Suppl 1)**:S21–S26. doi: 10.1007/s00420-002-0344-2.

31. Li DM, Han XD. Evaluation of toxicity of methyl tert-butyl ether (MTBE) on mouse spermatogenic cells in vitro. *Toxicol Ind Health*. 2006;**22(7)**:291–299. doi: 10.1177/0748233706070310.

32. Rekhadevi PV, Rahman MF, Mahboob M, Grover P. Genotoxicity in filling station attendants exposed to petroleum hydrocarbons. *Annals of Occupat Hyg*. 2010;**54(8)**:944–954. doi: 10.1093/annhyg/meq065.

33. Uzma N, Kumar BS, Hazari MAH. Exposure to benzene induces oxidative stress, alters the immune response and expression of p53 in gasoline filling workers. *Am J Ind Med*. 2010;**53(12)**:1264–1270. doi: 10.1002/ajim.20901.

34. Bryszewska M, Zavodnik IB, Niekurzak A, Szosland K. Oxidative processes in red blood cells from normal and diabetic individuals. *Biochem Mol Biol Int*. 1995; **37(2)**:345–354.

35. Arbos KA, Claro LM, Borges L, Santos CAM, Weffort-Santos AM. Human erythrocytes as a system for evaluating the antioxidant capacity of vegetable extracts. *Nutr Res*. 2008;**28(7)**:457–463. doi: 10.1016/j.nutres.2008.04.004.