

**BIOLOGICAL ASSESSMENT OF EXPOSURE TO
BENZENE AMONG PETROL STATIONS' WORKERS
IN ZAGAZIG CITY BY USING TRANS,
TRANS-MUCONIC ACID AS URINARY INDICATOR**

By

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Abstract:

Introduction: Benzene is an important raw material for the manufacture of synthetic rubbers, gums, lubricants, dyes, pharmaceuticals and agriculture chemicals. Occupational exposure to Benzene may induce some adverse health effects. **Aim of the work:** This study aimed to the evaluation of Benzene exposure among petrol Stations workers by assessment of some symptomatic, hematological and immunological changes by using trans- trans muconic acid (t, t MA) as a urinary indicator.

Subjects and Methods: A comparative cross – sectional study was conducted on five petrol stations. The study sample was 32 workers, they were classified as 12 exposed to benzene and 20 non-exposed control group. All subjects were interviewed and subjected to a structured questionnaire. They were asked to pass urine at the end of the shift to determine trans, trans muconic acid using high performance liquid chromatography (HPLC). Blood samples had obtained from all volunteers to detect peripheral blood mononuclear cells (PBMC) also serum concentrations of IgG and IgM were determined by suitable method. **Results:** A statistically significant higher prevalence of adverse symptoms among exposed workers compared to control group. In addition, there were significantly higher values of trans, trans muconic acid among exposed group

compared to control group with significant correlation ($P < 0.01$) between t, t MA level and duration of exposure. **Conclusion:** Exposure to benzene in Petrol stations workers induces significant reduction in peripheral blood mononuclear cells (PBMC), immunological changes, and adverse symptomatic effects.

Recommendations: More studies are needed to explore the details of occupational exposure to benzene for purposes of prevention and control.

Introduction:

Benzene is a ubiquitous indoor and outdoor pollutant causing occupational and public health problems (NICNAS 2001; WHO 2000) .

Benzene is a primary pollutant in the petrochemical and steel industry. Its production and consumption had caused world's wide concern for health. Risks resulting from exposure mainly because of its carcinogenic effect (Tsai et al., 2004).

Occupational exposure to benzene occurs mainly in the petrochemical industries, coke ovens and steel. Chemicals and associated industries, in laboratories using the chemical for research in analysis. Workers involve in the production, transportation and marketing of fuels are exposed to various levels of benzene (Skrobot et al., 2008).

Benzene is classified as a group I carcinogen by the International Agency For Research on Cancer and is listed by the World Health Organization (WHO, 2006) as a top priority compound.

Prolonged exposure to benzene causes various effects on human body especially myelotoxicity, genotoxicity and its carcinogenic actions. Other effects on various organs e.g. the central nervous system, the endocrine and immune system. Some studies showed that in addition to be a risk factor for leukemia, it can cause significant hematological changes in people exposed to even 1ppm (Lan et al., 2004).

The major sources of benzene in ambient air or urban are car exhaust and evaporation loses during handling, distribution and storage of petrol. (Carrier et al., 2006)

Urinary metabolites such as phenol, hydroquinone, trans, trans-muconic acid (t, t-MA), and S-phenol mercapturic acid are considered indices of occupational or environmental exposure to benzene (Boogaard & Sitter, 1996).

The American Conference of Governmental Industrial Hygienists (ACGIH) introduced (t,t-MA) as biological exposure index for benzene. It is a minor non-phenolic metabolite of benzene that

is excreted in urine (American Conference of Governmental Industrial Hygienists "ACGIH", 2003).

Aim of the study was the evaluation of Benzene exposure among petrol stations workers by measuring some symptomatic, hematological and immunological changes and using trans, trans Muconic acid as a urinary indicator.

Subjects and Methods:

This study was a comparative cross sectional study. It was carried out on a sample of workers exposed to benzene at petrol stations. Five stations were selected randomly from 20 stations at Zagazig City. 12 workers from these stations who are responsible for filling benzene at the station. They are exposed to benzene for at least 3 years. A control group consists of 20 non-exposed workers matched with the studied group as regard age and socioeconomic status.

An informed consent was provided to all participants illustrating the aim of the study and insuring complete confidentiality of the information acquired.

Simple structured questionnaire included socioeconomic characteristics work history and past medical history was filled from all participants.

I- t, t-MA in urine:

Exposed subjects and non-exposed control were asked to pass urine at the end of the shift. Samples were refrigerated immediately, transferred to the analytical laboratory and kept frozen until analysis.

The determination of t,t-MA was carried out according to the method of Boogaard & Sitter 1996

To improve the recovery, urinary samples were brought to pH 7-10 by the addition of 35% (w/v) sodium hydroxide aqueous solution before the sample was cleaned using solid phase extraction. Urinary samples were centrifuged (2.000 rpm for ten minutes) to separate suspended materials and 1ml was subsequently passed through a sax column, which had been previously conditioned with 3ml of acetonitrile and 3 ml of water. After washing with 3ml of 1%, percent acetic acid, t,t-MA was eluted from the cartridge with 4ml of 10% acetic acid. Twenty micro liters of this solution were analysed by high performance liquid chromatography (HPLC).

HPLC system equipped with a UV detector (HEWLEH Packar D) was used for analysis. The UV detector was set at 259 nm. The HPLC column was an APEX ODSII 3um (250 x 4.6mm) Beckman, USA analytical column.

Chromatography was isocratic in a mobile phase consisting of water-methanol-acetic acid (89: 10: 1).

The flow rate set at 1 ml /minute. All chemicals and water used were HPLC grade. In these condition, the retention time for t,t -MA is about 14-15 min.

The standard trans, trans-muconic acid (98%) was obtained from ALDRICH-Chemistry M90003-1G-07319CH, Sigma.

II- Methods for detection of (PBMC) and serum immunoglobulin IgG and IgM.

Blood samples were obtained from all volunteers 10 ml were collected in preservative free heparin and were used for analysis of (PBMC) 10 ml were centrifuged and sera were collected for IgG and IgM.

1. Method For Detection Of (PBMC):

After separation of mononuclear cells from heparinized peripheral blood by the method described by Boyum (1976), PBMC washed in phosphate buffered saline (pH 7.2) and cell concentrations were adjusted to 1×10^6 /ml.

Mononuclear cells were analyzed for cell surface phenotype by direct immune fluorescence technique. The numbers of PBMC were analyzed by flow cytometry.

2. Methods For Detection Of Serum Immunoglobulins IgG and IgM:

Serum concentration of IgG and IgM were determined by McLaren (1978), 50ul of predetermined optimal dilution of horse raddish peroxidase labeled antihuman IgG and IgM for human groups were added in micro ELISA plate with 100ul of the test sera diluted 1: 200, 100ul of one substrate working solution were added to each plate and was incubated at 37oC for 15-30 minutes.

The reaction was stopped by adding 20ul of 5 NH₂SO₄ to each well and the optical density was read at 492 nm using ELISA reader.

3- Methods For Detection T-Helper cells & T-suppressors CD4/CD8:

CD4/CD8 dual color Moab (Dako) was performed in EDTA anticoagulated peripheral blood in (PB) samples using FACS caliber flowcytometry (FCM).

CD4/CD8 dual color Moab were labeled with FITC (CD4) and PE (CD8). Samples were processed within 12 hours of collection. Whole blood technique was performed as 100ul of PB sample were incubated with an appropriate amount of the Moab (CD4/CD8) 15 min at 4oC. One ml lysing solution (1: 10) was added

for 10 mins. at room temperature and then centrifuged for 5 min at 1200 rpm. After two-cell washed with phosphate buffered saline. (PBS), the cell pellet was resuspended in 0.5 ml PBS. Ten thousands mononuclear cells were acquired by gating based on forward scatter and side scatter characteristics. Mouse IgG1 and IgG2 a (BD) were used as isotypic controls to determine background fluorescence (negative control). Data were analyzed with cell quest software (Becton Dickinson).

The diagnostic interpretation were based on the percentage of positive cells. To make the results comparable between different days. The sensitivity of fluorescence detectors and compensation were set and carefully monitored using calibrate feeds (BD).

Statistical Analysis :

All data were entered and analyzed using SPSS statistical software for windows. Differences between the mean values of the study variables were tested by the student's t test. Comparisons between proportions had done using Chi-square test.

Results:

The present work showed no statistical significant difference in the general characteristics regarding age, duration of exposure, residence either urban or rural among both exposed and non exposed studied groups $P > 0.05$ Table (1).

There was statistically significant higher prevalence of the symptoms like (drowsiness, dizziness, headache exhaust, tremors, sleepiness and confusion) among the exposed group compared to the control non exposed with $P < 0.05$ Table(2)

Regarding to blood parameters (Leukocytes, B-Lymphocytes, T Lymphocytes, T- Helper cells, T Suppressor- Natural Killer- IgG and IgM) had showed highly significant reduction among exposed group compared to the control group with $P \text{ value} < 0.001$ Table (3)

Regarding to the mean value of trans muconic acid showed statistically highly significant increment among exposed group (7.41 ± 2.73) compared to control group (1.04 ± 0.99) with $P < 0.001$ Table (4)

Table (1): General Characteristics of The Studied Groups.

| | <i>Exposed n=12</i> | <i>Control n=20</i> | <i>t</i> | <i>p</i> |
|------------------|---------------------|---------------------|-----------------------|----------|
| Age X±SD | 39.25 ± 5.53 | 37.15±5.49 | 1.04 | >0.05 |
| Duration X±SD | 7.16±2.91 | 6.05±2.78 | 1.08 | >0.05 |
| Residence (%) | | | | |
| Urban | 8 (66.6%) | 12 (60%) | X ² = 0.14 | >0.05 |
| Rural | 4 (33.4%) | 8 (40%) | | |

P>0.05= Non significant

Table (2): Prevalence of Symptoms Among The Studied Group.

| | | <i>Exposed n=12</i> | | <i>Control n=20</i> | | <i>t</i> | <i>p</i> |
|------------|---|---------------------|-------|---------------------|------|----------|----------|
| | | No | % | No | % | | |
| Drowsiness | + | 5 | 41.6% | 2 | 10% | 4.4 | <0.05 |
| | - | 7 | 58.4% | 18 | 90% | | |
| Dizziness | + | 5 | 41.6% | 2 | 10% | 4.4 | <0.05 |
| | - | 7 | 58.4% | 18 | 90% | | |
| Exhaustion | + | 4 | 33.3% | 1 | 5% | 4.56 | <0.05 |
| | - | 8 | 66.6% | 19 | 95% | | |
| Headache | + | 7 | 58.4% | 2 | 10% | 8.66 | <0.05 |
| | - | 5 | 41.6% | 18 | 90% | | |
| Tremors | + | 3 | 25% | 0 | 0% | 5.51 | <0.05 |
| | - | 9 | 75% | 20 | 100% | | |
| Confusion | + | 4 | 33.3% | 0 | 0% | 7.64 | <0.05 |
| | - | 8 | 66.6% | 20 | 100% | | |
| Sleepiness | + | 4 | 33.3% | 1 | 5% | 4.56 | <0.05 |
| | - | 8 | 66.6% | 19 | 95% | | |

P<0.05= Significant

Table (3): Profile of Blood Parameters among the Studied Group.

| <i>Parameters</i> | <i>Exposed n=12 X±SD</i> | <i>Control n=20 X±SD</i> | <i>t</i> | <i>P</i> |
|-----------------------------------|----------------------------------|----------------------------------|----------|----------|
| Leukocytes | 7832.75±818.75 | 8691.0±349.99 | -4.13 | <0.001 |
| Lymphocytes | 1886.5±232.27 | 2733.75±401.19 | -6.65 | <0.001 |
| B-lymphocytes | 471.33±58.14 | 683.15±100.30 | -6.64 | <0.001 |
| T-lymphocytes | 1320.25±162.55 | 1913.30±280.84 | -6.65 | <0.001 |
| T-Helper cells (CD ₄) | 725.58±98.48 | 1053.6±150.95 | -6.81 | <0.001 |
| T-suppressors (CD ₈) | 594.66±73.07 | 855.2±135.24 | -6.13 | <0.001 |
| Natural Killer NK | 94.25±11.37 | 137.15±21.16 | -6.45 | <0.001 |
| IgG | 503.33±17.54 | 622.8±14.49 | -6.61 | <0.001 |
| IgM | 70.58±8.67 | 85.15±3.73 | -19.81 | <0.001 |

Table (4): Mean Values of T, T-MA among the Studied Group.

| <i>Variable</i> | <i>Exposed n=12 X±SD</i> | <i>Control n=20 X±SD</i> | <i>t</i> | <i>P</i> |
|-----------------|----------------------------------|----------------------------------|----------|----------|
| t,t-MA | 7.41±2.73 | 1.04±0.99 | 9.48 | <0.001 |

P<0.001= Highly Significant

Discussion:

Benzene is one of the most widely used industrial chemicals. Studies on biological monitoring must consider the need for identifying suitable biomarkers that are sensitive, reliable and practical. Excretion of urinary t, t-MA has been recommended as a biomarker for estimation of occupational exposure to benzene and has been used in several surveillance studies (Ducos et al., 1992, Holz et al., 1995)

It is well established that the only significant source of t, t-MA formation in the body is through the metabolism of benzene, thus it is specific to benzene exposure (Johsen and Lucier, 1992).

Cigarette smoking is a potential confounding factor for low benzene monitoring, so in this study only the exposed non smokers were used for the analyses (Hajimiragha et al., 1989; Pekari et al., 1992).

Benzene is a radiomimetic chemical with heavy exposure resulting in progressive degeneration of the bone marrow, a plastic anemia, leukemia and dysfunction of the immune system (Yardley-Jones et al., 1991).

The present study revealed that petrol stations workers and the control group were matched as regard age, duration of work and

residence. Exposure to benzene reported high prevalence of symptoms as central nervous system symptoms that is consistent with solvent exposure including headache, fatigue, increased sleep requirement and confusion. Other researchers obtained the same results (Wiwanitkit, 2007). These symptoms were contributed to the aberration in basic electrophysiology of the brain and the vasomotor possible mechanism.

Our results revealed that exposure to benzene induce significant reduction of blood parameters as the means of peripheral blood lymphocytes and serum immunoglobulin (IgG and IgM) showed a highly significant reduction when compared with control group. This study correlated well with results in other studies of the effects of benzene exposure on lymphocytes (Lange et al., 1973). Several studies have demonstrated high lymphocyte sensitivity to benzene as they reported that lymphocytes are sensitive to toxic effects of benzene and benzene metabolites (Irons et al., 1983).

A study conducted by Mohamed et al., (1999) reported a significant reduction of both IgG and IgM in workers exposed to benzene for long period and explained their results by the autoimmune response, increased susceptibility to infections and loss of immunosurveillance.

The suitability of t,t-MA as a biomarker to monitor low exposure to benzene has been an issue since a few years (Hoet et al., 2009). This study indicated that exposed workers excreted significantly higher concentrations of t, t-MA than control group. A good correlation was found between t,t-MA and duration of exposure ($t=0.78$, $P<0.01$), suggesting that t,t-MA is specific to benzene exposure as indicated from previous studies (Johanson & Lucier, 1992).

A study was undertaken in Iran to evaluate exposure to benzene and the relationship between t,t-MA and atmospheric benzene among taxi drivers and petrol station workers, showed that the mean urinary t,t-MA in workers of petrol stations and drivers was less than gas station attendants. This is may explained by old motor technology and a lack of catalytic converters has led to unburned hydrocarbons being emitted into the ambient air of gasoline stations and also inside of vehicles, evaporation of volatile organics from car carburetors and petrol tanks, the relatively old age of cars and the consequent inefficiencies fuel burning of their motors (Bahrami et al., 2007).

Our study was consistent with previous studies in Italy (Fustinoni et al., 2005) who

found that excretion of t, t-MA measured at the end of the workshift showed increment

In conclusion, our results suggest that urinary, t, t-MA level was able to separate those exposed to benzene from non-exposed group. In addition, exposure to benzene can induce significant reduction in peripheral blood mononuclear cells (PBMC) and immunotoxic effects in petrol stations workers compared to control group.

Therefore, we recommend that extensive attention and periodic medical check for benzene exposure is in need for maintaining the health of to petrol stations workers.

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