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Xylene Induces Oxidative Stress and Mitochondria Damage in Isolated Human Lymphocytes

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Xylene is a cyclic hydrocarbon and an environmental pollutant. It is also used in medical technology, paints, dyes, polishes and in many industries as a solvent; therefore, an understanding of the interaction between xylene and human lymphocytes is of significant interest. Biochemical assessment was used to demonstrate that exposure of lymphocytes to xylene induces cytotoxicity (at 6 hr), generates intracellular reactive oxygen species, collapse of mitochondrial membrane potential, lysosomal injury, lipid peroxidation and depletion of glutathione (at 3 hr). The findings show that xylene triggers oxidative stress and organelle damage in lymphocytes. The results of our study suggest that the use of antioxidant, mitochondrial and lysosomal protective agents can be helpful for individuals subject to chronic exposure to xylene.

Key words: Xylene, Lymphocyte, Cytotoxicity, Mitochondrial damage, Oxidative stress

INTRODUCTION

Xylene is a cyclic hydrocarbon and an environmental pollutant, which is known by names such as dimethyl benzene and xylol (1). It is primarily used as a solvent in the printing, rubber, cleaning agent, paint thinner, varnish, synthetic fiber and leather industries. Contact with xylene occurs through the eyes and skin, orally and by inhalation. Occupations in which humans are exposed to xylene include histopathology laboratories, leather and rubber industries, petrochemicals and steel manufacturing (2-4).

Xylene has lipophilic properties. It is rapidly absorbed through all routes of exposure and quickly distributed throughout the body (2,5). Studies have shown that chronic occupational exposure to xylene is associated with a variety of hematological effects (6). Decreased white blood cell counts were observed in two women with chronic occupational exposure to xylene (7,8). Langman *et al*. found that chronic exposure to xylene is associated with thrombocytopenia, leukopenia and anemia. Occupational exposure to xylene significantly diminishes the percentage of lymphocytes in the peripheral blood (2,9). Studies have reported that children whose parents are exposed to xylene in the workplace are at high risk for leukemia, although the females generally showed normal blood parameters (10). The current study was designed to identify the cellular effects of xylene on lymphocytes in light of the findings of previous studies regarding the probability of decreased white blood cell counts in humans following exposure to xylene and the compound effect of ameliorating immune system activity against viral infection.

MATERIALS AND METHODS

Blood samples. All blood samples (n = 10) were acquired from Massoud Laboratory and approved by the Blood Administration Center of Tehran province in Iran. The studies were performed at the Faculty of Pharmacy at Shahid Beheshti University of Medical Sciences (Tehran, Iran) under the guidance of an expert physician. This study was approved by the Shahid Beheshti University of Medical Sciences research ethics committee and all participants signed informed consent forms (11).

Lymphocytes isolation. Lymphocytes were collected from healthy individuals who were 25 to 35 years of age.

Blood was obtained from 10 healthy, non-smoking volunteers who showed no signs of infection at the time the blood samples were collected. Lymphocytes were isolated using Ficoll-Paque Plus (GE, PA, USA) by centrifugation at 2,500 g for 20 min at 4°C. The lymphocytes were collected, suspended in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4) and incubated for 5 min at 37°C. PBS was added immediately and the cells were centrifuged at 1,500 g for 10 min at 4°C. The supernatant was decanted and the cells were washed twice with PBS at 2,000 g for 7 min. The cells then were resuspended in RPMI1640 medium with L-glutamine and 10% FBS. The final lymphocytes count used in the experiments was 1×10^6 cells/mL. The viability of the lymphocytes was over 95% (12).

Lymphocyte treatment. Commercial or mixed xylene usually contains 40% to 65% m-xylene and up to 20% each of o-xylene, p-xylene and ethylbenzene. The mixed xylene was first dissolved in 0.05% DMSO. The concentration of DMSO used in the experiments was non-toxic for lymphocytes as analyzed by cell viability and other parameters. The change in the untreated lymphocytes (negative controls) was 100%. The final concentration of the xylene used in the experiments was 50 to 2,000 ng/L. The cells were incubated in xylene for 6 hr to determine cell viability and oxidative stress mechanistic parameters within 3 hr of incubation. Incubation was carried out at 37°C in 5% CO_2 atmosphere (11).

Cell viability. Cell viability was assessed by MTT assay as described by Mosmann (13). In brief, lymphocytes (1×10^4 cells/well) were incubated in 96-well plates with and without xylene for 6 hr in a final volume of 10 μL. At the end of treatment, 25 μL of MTT (5 mg/mL in RPMI) was added to each well and incubated for an additional 1 hr at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 μL of DMSO and absorbance was measured at 570 nm with an ELISA reader. Each concentration was tested in three different experimental runs with three replicates per sample (12).

Reactive oxygen species assay. Reactive oxygen species (ROS) formation was measured at 1, 2 and 3 hr by DCFH-DA staining. In brief, 950 μL of cell suspension plus 50 μL of a specific concentration of xylene were incubated at 37°C in 5% CO_2 atmosphere in 24 plates. At the end of incubation, the cell wells were suspended and 300 μL was removed from each group. The removed cells were washed twice with PBS at 1,000 rpm for 5 min. After washing, the cells were resuspended in 300 μL of PBS containing 1.6 μM DCFH-DA and incubated for 15 min. Next, 100 μL of cells were diluted with deionized water in a cuvette and the fluorescence intensity of the DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. The excitation and emission wavelengths were 500 and 520 nm, respectively (14).

Mitochondrial membrane potential assay. Mitochondrial membrane potential (MMP) collapse was measured at 1, 2 and 3 hr by rhodamine 123 staining. MMP assay is similar to measurement of ROS except that, after washing, the removed cells were resuspended in 300 μL of PBS containing 1.5 μM rhodamine 123 and incubated for 15 min. Next, 100 μL of cells were diluted with deionized water in a cuvette and the fluorescence intensity of the rhodamine 123 was measured using a Shimadzu RF5000U fluorescence spectrophotometer. The excitation and emission wavelengths were 490 and 520 nm, respectively (14,15).

Lysosomal membrane integrity assay. Lysosomal membrane integrity was measured after 1, 2 and 3 hr by acridine orange staining. Lysosomal membrane integrity assay is similar to measurement of ROS except that, after washing the removed cells, they were resuspended in 300 μL of PBS containing 5 μM of acridine orange and incubated for 15 min. Next, 100 μL of cells were diluted with deionized water in a cuvette and the fluorescence intensity of the acridine orange was measured using a Shimadzu RF5000U fluorescence spectrophotometer. The excitation and emission wavelengths were 495 and 530 nm, respectively (14).

Lipid peroxidation assay. Lipid peroxidation was measured after 3 hr of exposure using the thiobarbituric acid reactive substances (TBARS) method. Briefly, 950 μL of cell suspension plus 50 μL of a specific concentration of xylene were incubated at 37°C in 5% CO_2 atmosphere in 24 plates. After exposure, 750 μL of the exposed cells were combined with 250 μL of 70% trichloroacetic acid (TCA) and 1 mL of distilled water was added. After 20 min, 1 mL of 0.8% thiobarbituric acid (TBA) was added. The mixture was placed in a boiling water bath for 20 min and then centrifuged at 13,000 rpm for 10 min. The amount of TBARS formed during the decomposition of lipid hydroperoxides was determined by following the absorbance at 532 nm (14,16).

GSH and GSSG. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to the spectrofluorometric method. Each sample was measured in quartz cuvettes using a fluorimeter set at 350 nm of excitation and a 420 nm emission wavelength (14).

Statistical analysis. The results are presented as mean ± SD. The assays were performed in triplicate and the mean was used for statistical analysis. Statistical significance was determined using one-way and two-way ANOVA, followed

by the post hoc Tukey and Bonferroni tests, respectively. Statistical significance was set at $p<0.05$.

RESULTS

Cell viability.   The results showed that xylene significantly reduced the viability of lymphocytes after 6 hr of incubation at concentrations of 1,000 to 2,000 ng/L. Fig. 1 shows xylene-induced cytotoxicity (28% and 38%) in the treated lymphocytes at 1,000 and 2,000 ng/L, respectively.

ROS measurement.   The results of ROS measurement showed that the amount of reactive oxygen radical formation after exposure to xylene increased significantly at concentrations of 500~2,000 ng/L after 1, 2 and 3 hr. Fig. 2A shows that Butylated hydroxytoluene (BHT) as an antioxidant significantly reduced xylene-induced blood lymphocyte ROS formation. H_2O_2 was used as a positive control for assessment of ROS formation. The effects of BHT, chloroquine and cyclosporine A (Cs.A) on the lymphocytes were separately tested (data not shown).

Malondialdehyde measurement.   Fig. 2B showed that a significant amount of TBARS formed only when human blood lymphocytes were incubated in xylene at a concentration of 2,000 ng/L. Xylene-induced TBARS formation was prevented by ROS scavenging (50 μM BHT), MPT pore sealing agent (5 μM Cs.A) and chloroquine, a lysosomotropic agent which inhibits a Fenton-type reaction. The effects of BHT, chloroquine and Cs.A on lipid peroxidation were separately tested (data not shown).
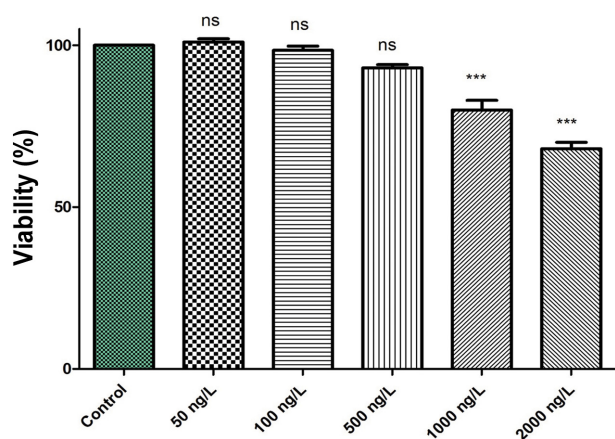
MMP assay.   The results showed that xylene at all time intervals (1, 2 and 3 hr) at concentrations of 500 and 2,000 ng/L significantly decreased MMP in the blood lymphocytes. Concurrent use of an antioxidant (BHT), lysosomal Fenton reaction inhibitor (chloroquine) and MPT pore-sealing agent (Cs.A) with xylene (1 μM) de- creased mitochondrial damage and inhibited MMP collapse. Ca^{+2} was used as a positive control for assessment of MMP collapse (Fig. 3). The effects of BHT, chloroquine and cyclosporine A on the MMP assay were separately tested (data not shown).
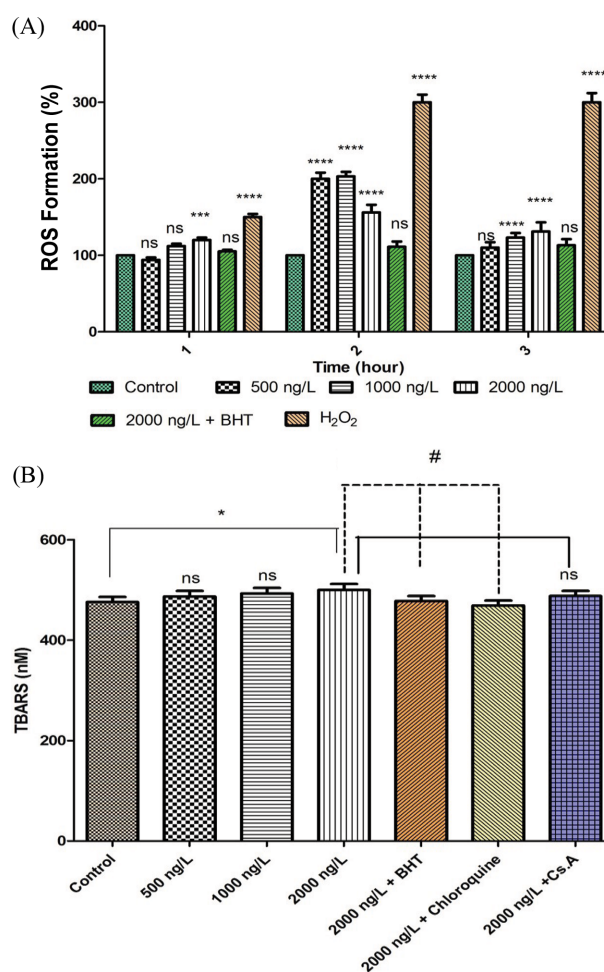

Fig. 2. Treated lymphocytes: (A) ROS formation; (B) lipid peroxidation. ROS formation was measured fluorometrically using DCF-DA. Lymphocytes (10^6 cells/mL) were incubated in RPMI 1640 at 37°C for 3 hr following addition of xylene. TBARS formation is expressed as nM. Values are expressed as mean ± SD of three separate experiments (n = 5). *Significant difference in comparison with untreated control ($p<0.05$). ***Significant difference in comparison with untreated control ($p<0.001$). ****Significant difference in comparison with untreated control ($p<.001$). #Significant difference in comparison with xylene-treated lymphocytes (2,000 ng/L) and preventive agents (BHT, chloroquine and Cs.A) ($p<0.05$).
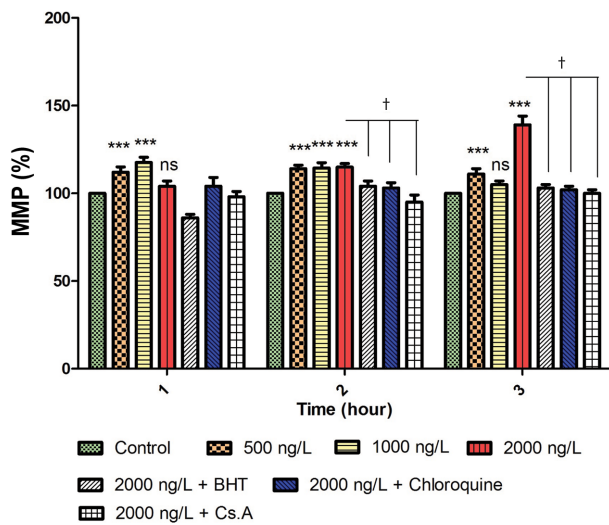

Fig. 1. Cell viability of lymphocytes from healthy donors seeded at 1×10^4 cells/well on 96-well plates, exposed to 50~2,000 ng/L xylene and then incubated for 6 hr. Absorbance representing the viability of the lymphocytes was determined by ELISA at 570 nm. Data presented as mean ± SD. The significance level was $p<0.05$ (n = 5). ***Denotes difference in comparison with untreated control ($p<0.001$). NS: Non-significant in comparison with untreated control.

Fig. 3. MMP declines in xylene-treated lymphocytes compared to those of untreated control. MMP collapse was measured by rhodamine 123. Cs.A and BHT inhibited the decline of MMP in lymphocytes exposed to 2,000 ng/L of xylene ($p<0.001$). Values represented as percentage of MMP decline ($n=3$). ***Significant difference in comparison with untreated control ($p<0.001$). †Denotes significant difference in comparison with xylene-treated lymphocytes ($p<0.05$).

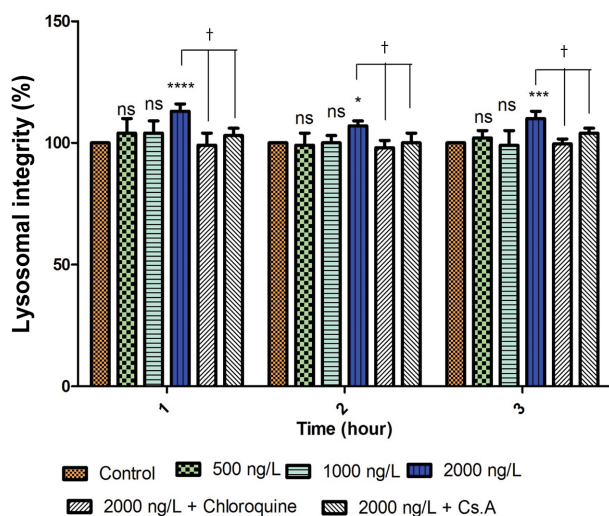**Fig. 4.** Lymphocytes (10^6 cells/mL) incubated in RPMI 1640 at 37°C. Lysosomal membrane damage was determined as the difference in redistribution of acridine orange from lysosomes into cytosol between the treated lymphocytes and untreated control lymphocytes. Results are shown as percentage of lysosomal membrane leakiness in all treated (test) lymphocyte groups. Values are expressed as mean ± SD of three separate experiments ($n=5$). The significant difference in comparison with the control lymphocytes was $p<0.05$. * Significant difference in comparison with untreated control ($p<0.001$). ***Denotes significant difference in comparison with xylene-treated lymphocytes ($p<0.001$). †Denotes significant difference ($p<0.05$) in comparison with lymphocytes treated with 2,000 ng/L of xylene.

Lysosomal membrane leakiness assay. The results showed that the highest concentration of xylene (2,000 ng/L) caused lysosomal membrane damage at 1, 2 and 3 hr. Concurrent use of an antioxidant (BHT), lysosomal Fenton reaction inhibitor (chloroquine) and MPT pore-sealing agents (Cs.A, carnitine) with 2,000 ng/L of xylene prevented lysosomal damage and rupture of the lysosomal membrane (Fig. 4). The effects of BHT, chloroquine and Cs.A on the lysosomal membrane integrity were separately tested (data not shown).

GSH and GSSG measurement. Fig. 5 shows that incubation of lymphocytes in xylene at concentrations of
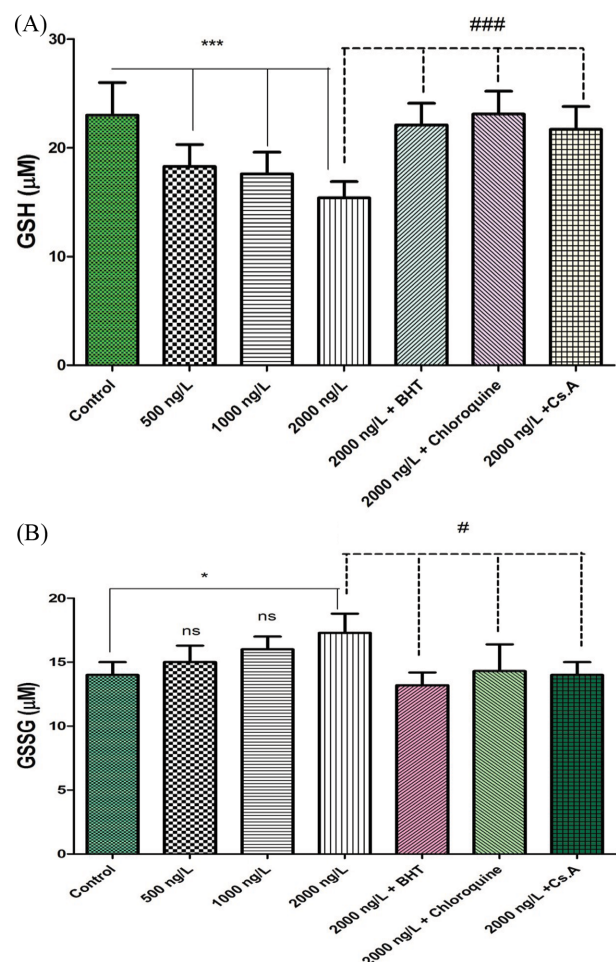

Fig. 5. Lymphocytes (10^6 cells/mL) incubated in RPMI 1640 at 37°C for 3 hr following the addition of xylene. Fluorometrically determined: (A) intracellular GSH and (B) extra cellular GSSG. Values are expressed as mean ± SD of three separate experiments ($n=5$). *Significant difference in comparison with untreated control ($p<0.05$). ***Denotes significant difference in comparison with untreated control ($p<0.001$). #Denotes significant difference in comparison with xylene-treated lymphocytes and preventive agent ($p<0.05$). ###Significant difference in comparison with 2,000 ng/L ($p<0.001$).

500~2,000 ng/L induced rapid lymphocyte intracellular GSH depletion (Fig. 5B). Most of the xylene-induced GSH depletion can be attributed to the expulsion of GSSG (Fig. 5B). Again, addition of an antioxidant (BHT), MPT pore-sealing agents (Cs.A and carnitine) and a lysosomotropic agent (chloroquine) significantly prevented a decrease in xylene-induced intracellular GSH and an increase in extracellular GSSG. None of these reagents produced a significant effect on lymphocyte GSH/GSSG status at the concentrations used (data not shown).

DISCUSSION

Decreased lymphocyte and serum complements were detected in workers who had been heavily exposed to xylene (8). Studies reveal adverse effects such as a decrease in the weight of the thymus and spleen in rats exposed to 2,000 mg/kg of xylene daily (1). The results of the current study showed that xylene is toxic to human lymphocytes at 1,000 and 2,000 ng/L after 6 hr of exposure. Studies on humans and animals have shown that xylene is well-absorbed by inhalation and oral routes. In the blood, it is primarily bound to serum proteins and accumulates in adipose tissue (17). Xylene is very soluble in blood and therefore absorbed easily into systemic circulation after exposure. This means that it is likely to attain such high concentrations after chronic exposure (18). Interestingly, the toxic concentrations obtained in the current results were similar to the human exposure levels found by Perbellini *et al* (19).

Studies have found that the toxicity of many organic compounds appear to relate to the induction of ROS through systemic elevation of the normal cellular rate of oxygen radical generation (20-22). Studies have revealed that ROS are generated during metabolism of xylene (23). It was also recently reported that o-xylene can induce toxicity and apoptosis individually in *Drosophila melanogaster* and human leukemia cells by increasing ROS production (20,23). In the present study, ROS production was altered in cells treated with xylene.

Previous investigations have shown that xylene is a more effective uncoupling agent and more effective in depleting ATP (24). Xylene is an aromatic hydrocarbon without proton releasing groups; therefore, rather than a protonophoretic mechanism, it binds to membrane hydrophobic sites such as proteins, an event which enhances its flux and/or interaction with the mitochondrial membrane targets (24). Revilla *et al*. found that xylene was capable of eliciting significant mitochondrial swelling in apparent association with increased ROS generation (24). The current results indicate that at the concentrations used, xylene could induce MMP collapse and increase ROS generation. The latter was inhibited by BHT as an antioxidant agent, which reflects the role of ROS formation in mitochondrial damage.

Hydrogen peroxide is mainly produced by the mitochon-

dria and diffuses into lysosomes in abnormal amounts. Because many lysosomes are rich in redox-active Fe^{2+}/Fe^{3+} due to degradation of iron-containing macromolecules, Fenton-type reactions then take place and result in lysosomal membrane rupture with the release of powerful lytic enzymes (25). The results of the current study showed that the use of antioxidant and MPT pore-sealing agents inhibited lysosomal damage.

In recent years, it has been suggested that lipid peroxidation plays an important role in toxicity induced by organic solvents (26). The results of the present study showed that xylene treatment of lymphocytes at 2,000 ng/L provoked potentiation of lipid peroxidation in the membranes of lymphocytes. It has also been suggested that xylene-induced lipid peroxidation may be a consequence of impairment of antioxidant defense systems such as glutathione and glutathione-related enzymes. The current study confirms these findings. It was shown that changes in the cellular defense system (decreased GSH content and increased GSSG) were associated with mitochondrial damage and ROS formation.

The current study reveals that xylene is toxic to human lymphocytes through ROS formation and mitochondrial and lysosomal damage that results in oxidative stress, depletion of GSH and cytotoxicity. Moreover, the current results suggest that the use of antioxidants can help protect individuals who are at risk of exposure to industrial solvents like xylene.

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

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