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RESEARCH ARTICLE

Toxicity of methyl tertiary-butyl ether on human blood lymphocytes

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Abstract Methyl tertiary-butyl ether (MTBE) is a synthetic solvent widely used as oxygenate in unleaded gasoline. Few studies have addressed the cellular toxicity of MTBE on some cell lines, and so far, no comprehensive study has been conducted to investigate the probable immunotoxicity of this compound. In this study, the toxicity of MTBE on human blood lymphocytes was evaluated. Blood lymphocytes were isolated from healthy male volunteers' blood, using Ficoll polysaccharide followed by gradient centrifugation. Cell viability, reactive oxygen species (ROS) formation, lipid peroxidation, glutathione levels, and damage to mitochondria and lysosome were determined in blood lymphocytes after 6-h incubation with different concentrations of MTBE (0.1, 0.5, 1, and 2 mM). Our results showed that MTBE, in particular, decreased cell viability, which was associated with significant increase at intracellular ROS level and toxic alterations in mitochondria and lysosomes in human blood lymphocytes. Moreover, it was shown that MTBE strongly provoked lipid peroxidation and also depleted glutathione level at higher concentrations. Interestingly, MTBE exhibited its cytotoxic effects at low concentrations that may resemble to its concentrations in human blood following occupational and environmental exposure. It is therefore concluded that MTBE was capable of inducing oxidative stress and damage to mitochondria and lysosomes in human lymphocytes at concentrations ranging from 5 to 40 μg/L, which may be present in human blood as a result of environmental exposure.

Keywords Methyl tertiary-butyl ether · Mitochondria · Oxidative stress · Cytotoxicity · Glutathione · Lysosomes

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Introduction

Methyl tertiary-butyl ether (MTBE) belongs to a large family of oxygenated ethers such as methyl tertiary amyl ether and ethyl tertiary butyl that came into use as motor vehicle fuel additives in the late 1970s with a significant increase in the 1990s (Sgambato et al. 2009). MTBE is used as an additive to oxygenate gasoline to improve air quality by reducing tailpipe emissions of ozone precursors and carbon monoxide. Due to its enormous production, widespread use, and improper disposal of used motor oil, MTBE has been found in urban storm runoff and streams, lakes, and groundwater. Then, potential for human exposure is high (Dong-Mei et al. 2009). MTBE pollution is considered as a critical environmental problem. It is often found at contaminated sites and is thought to be harmful to human health. Exposure to MTBE has been a long time concern in many occupational fields, including gasoline transport by tanker lorries, service stations, policemen, and the petroleum industry. Traffic policemen and gas station workers are two of the groups most heavily exposed to vehicle exhausts during their work shifts (Egeghy et al. 2000, Crebelli et al. 2001, Scibetta et al. 2004). According to literature, in estimation of workers' exposure with biological samples,

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analysis of the concentration of MTBE in urine detected after the work shift seems to give the most reliable estimation of exposure (Saarinen et al. 1998, Hakkola et al. 2001, Vainiotalo et al. 2006). Increasing use of these oxygenates together with concerns about the effects of oxygenated fuels on human health has prompted research efforts in several fields (Nihlén et al. 1998, Alimohammadi et al. 2005, Khavanin et al. 2005, Nikpey et al. 2006, Tomei et al. 2006, Silva et al. 2008, Dong-Mei et al. 2009). Many in vitro and in vivo studies have reported the adverse effect of MTBE on experimental animals (Li et al. 2007, de Peyster et al. 2008, Dong-Mei et al. 2009, Li et al. 2009, Pourahmad et al. 2012), while there was not wide evidence to show that the uses of MTBE have imposed significant harmful effects on human health. Acute exposures to MTBE have reported to cause a variety of symptoms including nausea; headaches; nose, throat, and eye irritation (Fiedler et al. 2000). Some animal exposure studies have also reported the carcinogenicity of MTBE. Environmental Protection Agency (EPA) has classified MTBE as a potential human carcinogen. Besides, several toxicity studies related to MTBE revealed that MTBE could exert toxic effects on cultured spermatogenic cells (Li and Han 2006, Li et al. 2007), cause DNA damage on mice fibroblasts (Song et al. 2002), and induce cell transformation in cultured rodent fibroblasts (Iavicoli et al. 2001).

Peripheral blood lymphocytes are exposed to gases and particulate matter present in the atmosphere. Exposure to polluted air containing MTBE is therefore suspected to induce damage to human blood lymphocytes. Immunotoxicity studies on rats have shown that fuel oxygenates including MTBE have toxic effect on immune system (White et al. 2014). Addition to in vivo study performed on rats has shown that MTBE exerted toxicity on white blood cell count, including lymphocyte, granulocyte, and eosinophil (Dong-Mei et al. 2009). Human study among gasoline station attendants indicated that immune reactions to MTBE do occur through hapten carrier reactions which, in some individuals, end with specific 1gG and IgM production (Vojdani et al. 1997b). The current study was therefore designed to evaluate whether isolated blood lymphocytes obtained from healthy donors are vulnerable to exposure to MTBE concentrations. The second objective of this study was to investigate the cytotoxicity mechanisms involved in possible MTBE-induced human blood lymphocytes.

Materials and methods

Chemicals

Rhodamine 123, collagenase, bovine serum albumin (BSA), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), acridine orange, 2′,7′-dichlorofuorescin diacetate (DCFH-DA), trichloroacetic acid, trypan blue, MTBE, and RPMI 1640 were purchased from Sigma-Aldrich Co. (Taufkirchen, Germany). All other chemicals were of the highest commercial grade available.

Blood samples and ethics statement

All blood samples ($n = 25$) were acquired from Massoud Laboratory and approved by Blood Administration Center of Tehran Province. The studies were performed at the Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, under the guidance of an expert physician. This study was approved by the Shahid Beheshti University of Medical Sciences research ethics committee, and all healthy donors signed an informed consent form.

Lymphocyte isolation

Lymphocytes were collected from healthy donor at age range 25 to 35 years old. Blood was obtained from 25 healthy, non-smoking volunteers, who showed no signs of infection disease symptoms at the time that the blood samples were collected. Lymphocytes were isolated using Ficoll Paque Plus by centrifugation at 2500*g* for 20 min at 25 °C. Lymphocytes were collected, suspended in erythrocyte lysis buffer (150 mM NH4Cl, 10 mM NaHCO3, 1 mM EDTA, 183 pH 7.4), and incubated for 5 min at 37 °C. Then, PBS was added immediately, and the cells were centrifuged at 1500*g* for 10 min at 20 °C. The supernatant was decanted, and the cells were washed twice with RPMI with L-glutamine and 10 % fetal bovine serum (FBS) at 2000*g* for 7 min. The cells were resuspended in RPMI medium with L-glutamine and 10 % FBS and counted in hemocytometer. The final lymphocyte density used in the experiments was 1×10^6 cells/ml. The viability of the cells was over 95 %.

Lymphocyte treatment

MTBE dissolved in DMSO. The final concentration of DMSO in negative control samples (without MTBE) and samples treated with individual MTBE was 0.05 % during analysis of all parameters. The concentrations of DMSO used in the experiments (samples) were not toxic for lymphocytes as analyzed by cell viability and other parameters. The changes in untreated cells (negative controls) were referred to as 100 %. The final concentrations of the compounds used in the experiments were in the range from 0.1, 0.5, 1, and 2 mM. The cells were incubated with MTBE for 6 h to analyze cell viability, and oxidative stress mechanistic parameters were studied within 3 h of incubation. The incubation was performed at 37 °C in 5 % CO_2 atmosphere in the total darkness.

Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann (1983). The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The dehydrogenases using NADH or NADPH as co-enzyme can convert the yellow form of the MTT salt to insoluble, purple formazan crystals (Liu et al. 1997). Formazan solution is read spectrophotometrically after the crystals are dissolved in organic solvent (DMSO). Lymphocytes (1×10^4cells/well) were incubated in 96-well plates in the presence or absence of MTBE for 6 h in a final volume of 50 μl. At the end of the treatment, 25 μl of MTT (5 mg/ml in RPMI) was added to each well and incubated for an additional 2 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO, and the absorbance was measured at 570 nm with ELISA reader. Each concentration was tested in three different experimental runs in three replicates for each sample.

Determination of reactive oxygen species

To determine the rate of lymphocyte reactive oxygen species (ROS) generation induced by MTBE, dichlorofluorescein diacetate (DCFH-DA, 1.6 μM) was added to the lymphocytes. It penetrates PBMC cells and becomes hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The latter then reacts with ROS to form the highly fluorescent dichlorofluorescein (DCF), which effluxes the cell. The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Pourahmad et al. 2011a).

Mitochondrial membrane potential assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123 (1.5 μM), has been used for the determination of mitochondrial membrane potential in human blood lymphocytes (Pourahmad et al. 2009). The amount of rhodamine 123 remaining in the incubation medium was measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 490-nm excitation and 520-nm emission wavelengths. The capacity of mitochondria to uptake the rhodamine 123 was calculated as the difference (between control and treated cells) in rhodamine 123 fluorescence (Rezaei et al. 2008).

Lysosomal membrane integrity assay

Lymphocyte lysosomal membrane stability was determined from the redistribution of the fluorescent dye, acridine orange, from lysosomes into cytosol (Pourahmad et al. 2010). Aliquots of the cell suspension (0.5 ml) that were previously stained with acridine orange (5 μM) were separated from the incubation medium by 1-min centrifugation at 1000 rpm. The cell pellet was then resuspended in 2 ml of fresh incubation medium. This washing process was carried out twice to remove the fluorescent dye from the media. Acridine orange redistribution in the cell suspension was then measured fluorimetrically using a Shimadzu RF5000U fluorescence spectrophotometer set at 495-nm excitation and 530-nm emission wavelengths. Lysosomal membrane damage was determined as the difference in redistribution of acridine orange from lysosomes into cytosol between treated lymphocytes and control lymphocytes at the time of preparation (Pourahmad et al. 2011b; Pourahmad et al. 2012).

Lipid peroxidation

Evaluation of lipid peroxidation in lymphocytes was conducted by determination of the amount of thiobarbituric acid reactive substances (TBARS) formed during the decomposition of lipid hydroperoxides by following the absorbance at 532 nm in a Beckman DU-7 spectrophotometer (Seydi et al. 2015).

GSH and GSSG

Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined according to the spectrofluorometric method (Hissin and Hilf 1976). Each sample was measured in quartz cuvettes using a fluorimeter set at 350-nm excitation and 420-nm emission wavelengths (Pourahmad et al. 2000).

Statistical analysis

Results are presented as mean ± SD. Assays were performed in triplicate, and the mean was used for statistical analysis. Statistical significance was determined using the one-way and two-way ANOVA tests, followed by the post hoc Tukey and Bonferroni tests, respectively. Statistical significance was set at $P < 0.05$. In all graphs, the results were expressed as mean ± SD and $P < 0.05$ was considered statistically significant.

Results

Cell viability

For determination of cytotoxicity of MTBE on lymphocytes, we used MTT assay. Our results showed that even at the lowest concentration (0.1 mM), MTBE has toxic effect toward lymphocytes. As shown in Fig. 1, MTBE at concentrations of 0.1, 0.5, 1, and 2 mM significantly ($P < 0.001$) reduced cell

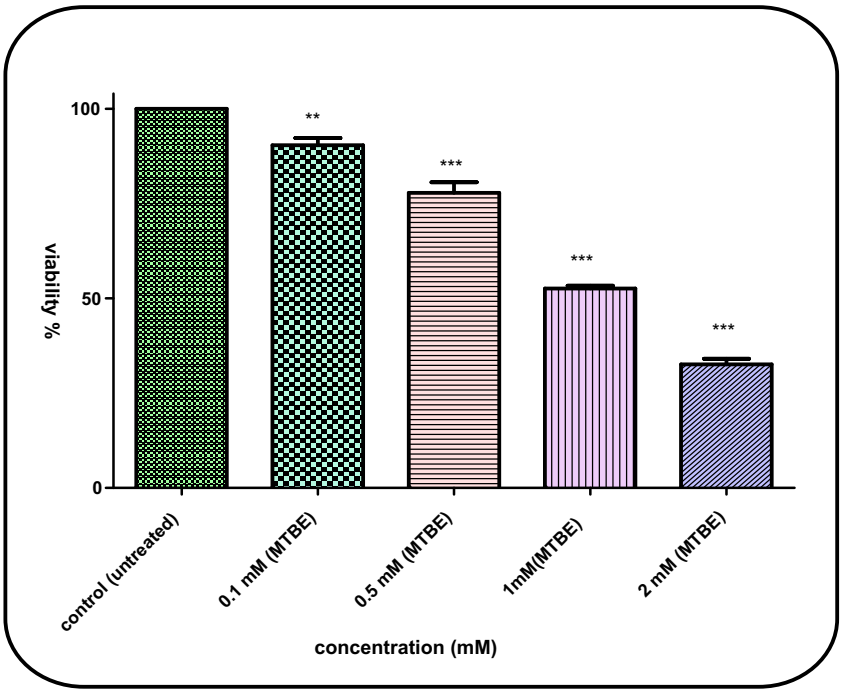

Fig. 1 Determination of cell viability in MTBE-treated human lymphocytes. Lymphocytes from healthy donors, at 1×10^4 cells/well, were seeded on 96-well plates. MTBE at 0.1-, 0.5-, 1-, and 2-mM concentrations was incubated in human lymphocytes for 6 h. The absorbance representing the viability decrease in lymphocytes was determined by the ELISA reader at 570 nm. Data presented as mean ± SD ($n = 5$). The significant level was set at $P < 0.05$

viability down to 9.4, 22, 47, and 61 %, respectively (Fig. 1). The IC_{50} measured for MTBE on lymphocytes was 1 mM.

Determination of ROS

In our study, we tested the effect of MTBE on ROS formation at time intervals (30, 120, and 180); we used 0.1-, 0.5-, and 1-mM concentration of MTBE.

Besides, when human blood lymphocytes were incubated with MTBE at different concentrations of 0.1, 0.5, and 1 mM, ROS formation (determined by the oxidation of dichlorofluorescein to dichlorofluorescein) was also significantly ($P < 0.05$) raised. MTBE (0.5 mM)-induced ROS formation was significantly prevented by butylated hydroxytoluene (BHT) (Fig. 2).

Determination of MMP

Mitochondrial membrane potential, which is a highly sensitive indicator of the mitochondrial inner membrane condition, was measured by rhodamine 123 redistribution. As shown in Fig. 3, matrix metalloproteinase (MMP) significantly ($P < 0.05$) decreased in lymphocytes of MTBE-treated group compared with untreated control group (Fig. 3). The mitochondrial membrane potential in two groups was measured in the time intervals of 30, 120, and 180 min. Furthermore, 5 μM cyclosporine A (an MPT blocker) and 50 μM BHT as an antioxidant strongly inhibited the decline of MMP in lymphocytes exposed to 0.5 mM of MTBE ($P < 0.05$). An MPT inducer, $CaCl_2$ (50 μM), was also used as a positive control in MMP assay (Fig. 3).

Lysosomal membrane integrity assay

When lymphocytes lysosomes were loaded with acridine orange (a lysosomotropic agent), a significant release of acridine orange ensued into the cytosolic fraction within 180 min of incubation with MTBE indicating severe damage to lysosomal membrane (Fig. 4). MTBE -induced acridine orange release was again prevented by BHT and chloroquine (100 μM). All of these reagents did not induce any marked effects on acridine orange redistribution from lysosomes to cytosol at concentrations used (data not shown).

Lipid peroxidation

As shown in Fig. 5, a significant amount of TBARS was formed when incubated with different concentrations of MTBE (0.1, 0.5, and 1 mM). MTBE-induced TBARS formation was prevented by ROS scavenger (BHT 50 μM) and MPT pore sealing agent (Cs.A 5 μM). MTBE-induced lipid peroxidation was also inhibited by GSH (Fig. 5).

GSH and GSSG

As shown in Fig. 6, incubation of lymphocytes with MTBE produced rapid lymphocytes GSH depletion. Most of the MTBE-induced GSH depletion may be attributed to the expulsion of GSSG (Fig. 6b). Again, antioxidant (BHT), MPT

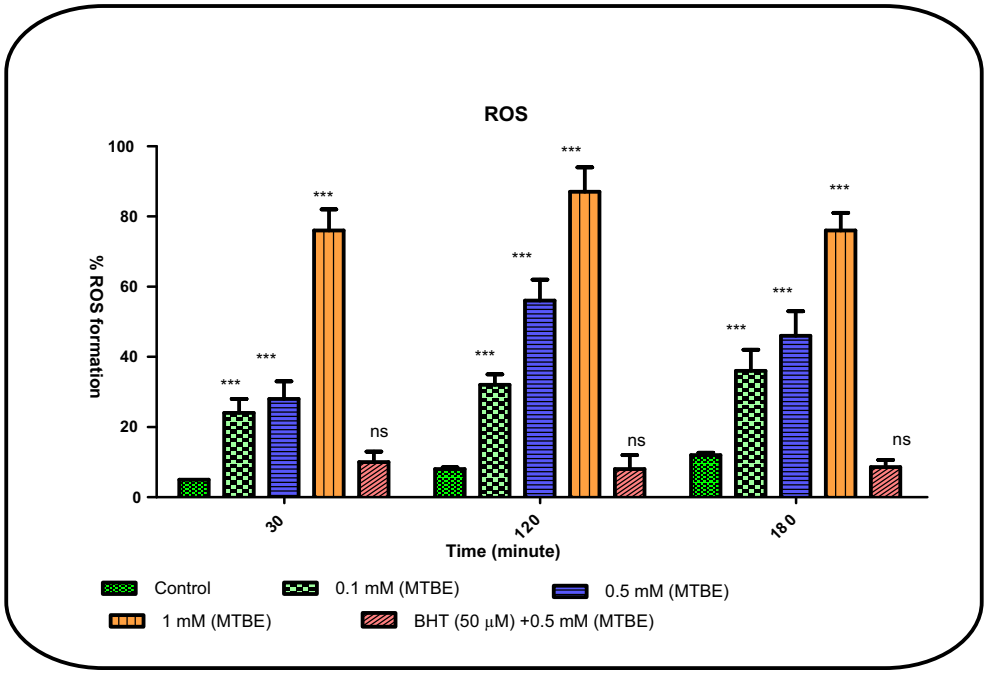

Fig. 2 Determination of ROS formation in MTBE-treated human lymphocytes. ROS formation was measured fluorometrically using DCF-DA as described in "Materials and methods" section. Values are presented as mean ± SD ($n = 3$). ***$P < 0.0001$ significant difference compared to untreated control

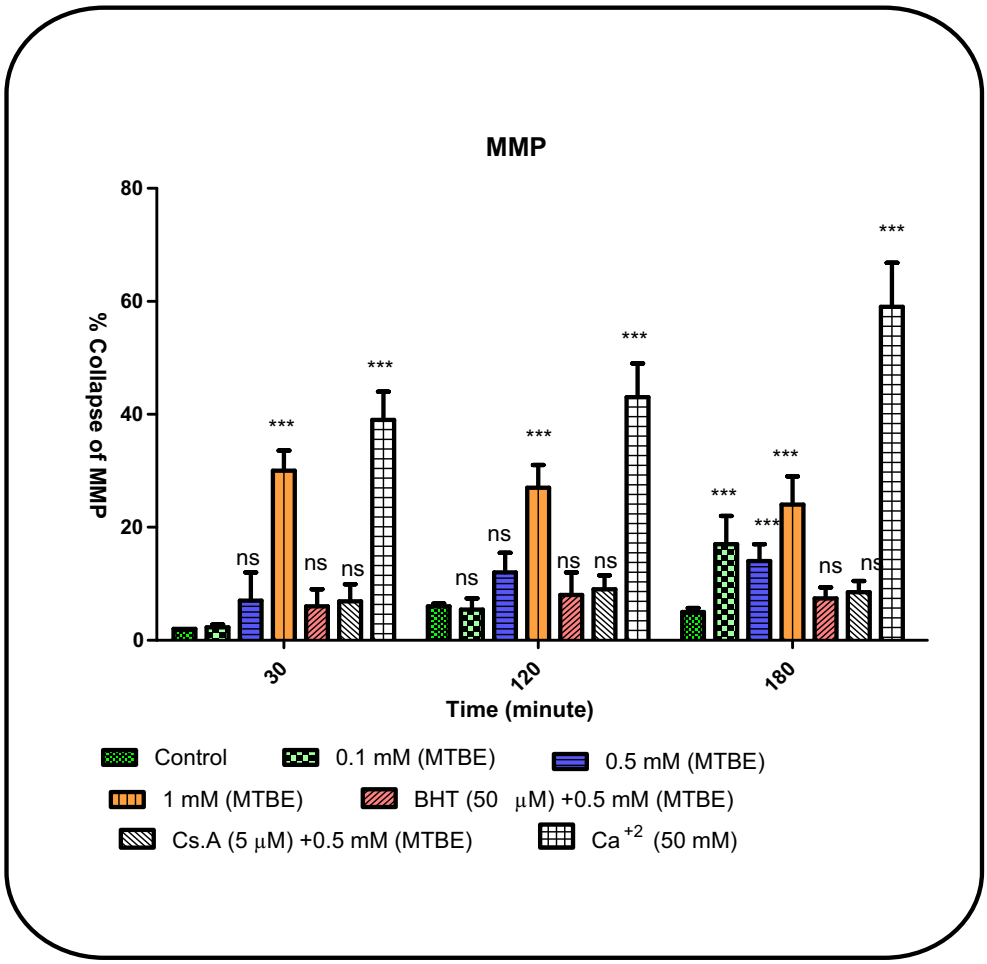

Fig. 3 Determination of MMP collapse in MTBE-treated human lymphocytes. Collapse of MMP was measured by rhodamine 123 as described in "Materials and methods" section. Cs.A and BHT inhibited the decline of MMP in lymphocytes exposed to MTBE (0.5 mM) ($P < 0.05$). Ca^{+2} was used as a positive control in MMP assay. Values represented as percentage of mitochondrial membrane potential decline ($n = 3$). ***Significant difference ($P < 0.0001$) compared to untreated control

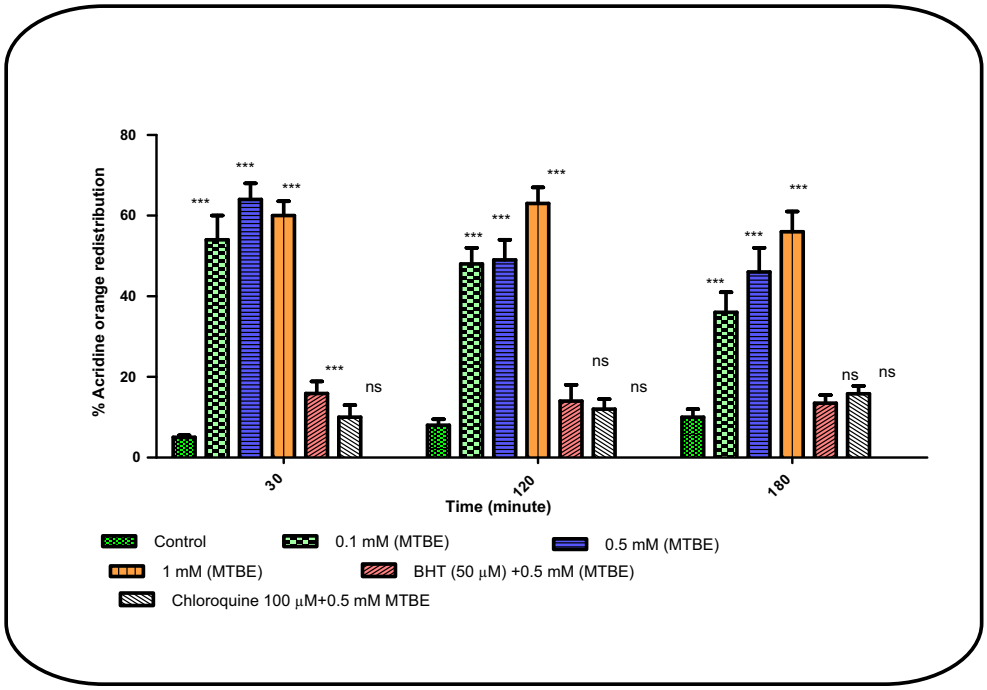

Fig. 4 Determination of lysosomal membrane damage in MTBE-treated human lymphocytes. Lysosomal membrane damage was determined as the difference in redistribution of acridine orange from lysosomes into cytosol between the treated lymphocytes and untreated control lymphocytes. Results were shown as % lysosomal membrane leakiness in all treated (test) lymphocyte groups. Values are expressed as mean ± SD of three separate experiments ($n = 3$). ***Significant difference in comparison with control lymphocytes ($P < 0.001$). ***Significant difference in comparison with MTBE-treated lymphocytes ($P < 0.001$)

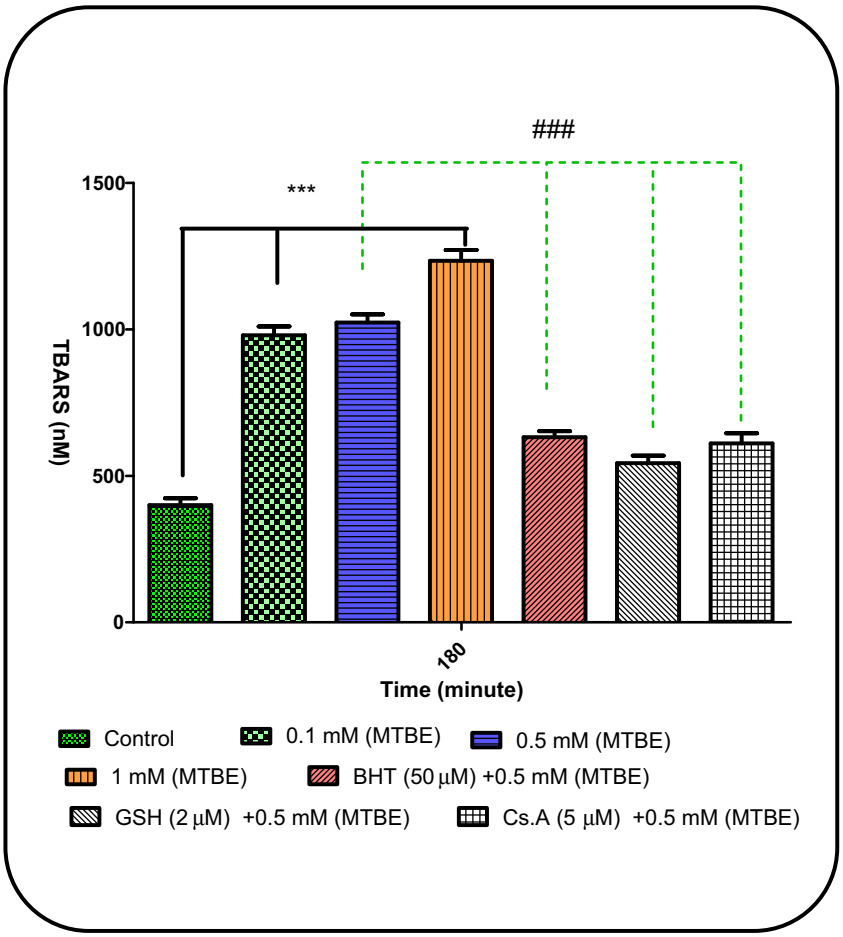

Fig. 5 Determination of lipid peroxidation in MTBE-treated human lymphocytes. Lymphocytes (10^6 cells/ml) were incubated in the RPMI 1640 at 37 °C for 180 min following the addition of MTBE. TBARS formation was expressed as nanomolar concentrations. Values are expressed as mean ± SD of three separate experiments ($n = 5$). ***Significant difference in comparison with untreated control lymphocytes ($P < 0.001$). ###Significant difference in comparison with MTBE-treated lymphocytes (0.5 mM) and preventive agent (BHT, GSH, and Cs.A) ($P < 0.001$)

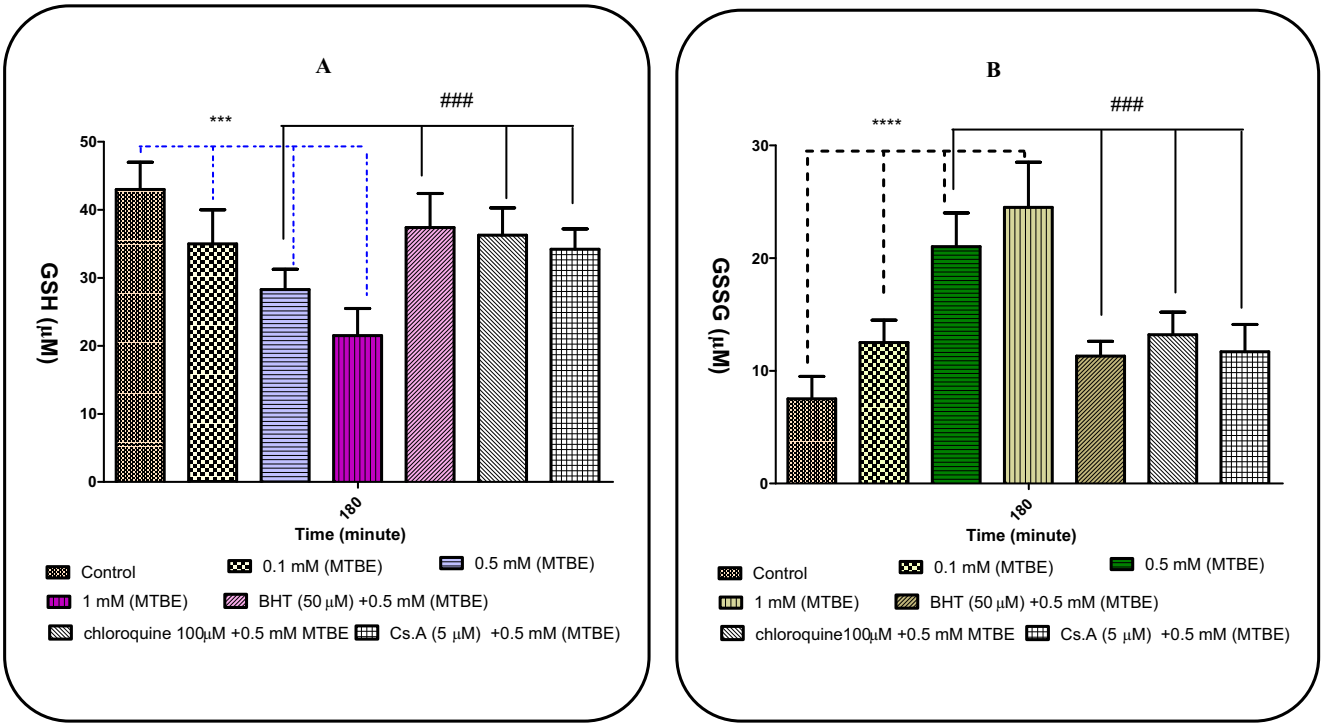

Fig. 6 Determination of intracellular GSH and extracellular GSSG in MTBE-treated human lymphocytes. Lymphocytes (10^6 cells/ml) were incubated in the RPMI 1640 at 37 °C for 180 min following the addition of MTBE. Intracellular GSH (**a**) and extracellular GSSG (**b**) were fluorimetrically determined. Values are expressed as mean ± SD of three separate experiments ($n = 5$). ***Significant difference in comparison with untreated control ($P < 0.001$). ###Significant difference in comparison with MTBE-treated lymphocytes and preventive agent ($P < 0.001$)

pore-sealing agents (Cs.A), lysosomotropic agents (chloroquine) significantly prevented both MTBE-induced intracellular GSH decrease and extracellular GSSG increase (Fig. 6). All of these reagents did not produce any significant effects on lymphocytes GSH/GSSG status at concentrations used (data not shown).

Discussion

Considering the fact those potential toxic effects of chemicals on the immune system arises from the critical role of this system in maintaining health. It is well recognized that suppressed immunological function can result in increased incidence and severity of infectious diseases as well as some types of cancer (Reiche et al. 2004). Quite in contrast, inappropriate enhancement of immune function or the generation of misdirected immune responses can precipitate or exacerbate development of allergic and autoimmune diseases (Griem et al. 1998). Thus, both suppression and enhancement of immune function are considered to represent potential immunotoxic effects of chemicals (Gleichmann et al. 1989). The aim of this study was to search the possible toxic effect of MTBE on blood lymphocytes that are important components in immune system. In this investigation the cellular toxicity endpoints such as viability decrease, lipid peroxidation, increased ROS formation, collapse of MMP and lysosomal membrane leakiness were tested in human blood lymphocytes following the addition of different concentrations of MTBE. Moreover, the effect of this substance on anti oxidant status most importantly intracellular GSH and extracellular GSSH levels was assessed.

According to our knowledge, no other comprehensive research has so far been conducted to evaluate the possible toxicity of MTBE on human lymphocytes and its mechanisms, in spite of some laboratory reports, which suggested that MTBE may affect the blood cells either by stimulating or inhibiting the activity of some cellular components (Pleil 2009).

We observed that MTBE caused statistically significant decrease in cell viability even at 0.1 μM after 6 h of incubation, which showed its cytotoxic potential towards human blood lymphocytes (Fig. 1). Previous studies also showed that MTBE could cause cytotoxic effect on several cell lines (Li et al. 2007).

Our findings regarding oxidative stress parameters revealed that MTBE could increase ROS production and enhance lipid peroxidation (Figs. 2 and 5). The increased ROS production may play a significant role in the enhancement of lipid peroxidation and finally cellular and sub cellular membrane damage.

Mitochondria are deeply involved in the production of ROS through one-electron carriers in the respiratory chain; mitochondrial structures are also very susceptible to oxidative stress. Our results proved that MTBE could cause mitochondrial damage which could be prevented by antioxidant (BHT) and MPT pore-sealing agents (Cs.A) (Fig. 3). Also mitochondria damages may lead to release of proapoptotic agents from mitochondria to cytosol and finally induce apoptosis through mitochondria pathways related to oxidative stress induced by MTBE. Moreover previous study on human blood lymphocyte by Vojdani et al. showed apoptotic effect (Vojdani et al. 1997a). GSH is an intracellular antioxidant that prevents an intracellular ROS formation and lipid peroxidation. As an antioxidant, it has been involved in cell protection from deleterious effects of the oxidative stress, both directly and as a cofactor of glutathione peroxidases, and these reactions generate GSSG (Pompella et al. 2003). Thus, the GSH depletion is a marker of the cellular oxidative stress and may be attributed to the expulsion of GSSG. Our results showed that when lymphocytes were incubated with MTBE, the GSH depletion occurred as a consequence of the ROS formation and the lipid peroxidation. These results are agreement with previous study (Pourahmad et al. 2012) suggesting that the MTBE-induced toxicity was related to decrease in the GSH content.

When lymphocyte lysosomes were loaded with acridine orange, a massive redistribution of acridine orange into the cytosolic fraction ensued within 180 min when the loaded lymphocytes were treated with MTBE, indicating leakiness of the lysosomal membrane (Fig. 4). On the other hand, the MTBE-induced acridine orange release was prevented by the lysosomotropic agent (chloroquine) and a ROS scavenger (BHT), suggesting involvement of pH-dependent intralysosomal Fenton's type reactions and ROS formation in MTBE-induced lysosomal membrane damage in human lymphocytes.

All our data was in favor of the involvement of the oxidative stress hypothesis in MTBE-induced human blood lymphocyte cytotoxicity, since both the decline in MMP and lysosomal membrane labilization were ensued following the MTBE-induced ROS formation, the lipid peroxidation, and the GSH depletion in blood lymphocytes. It is assumed that the oxidative stress causes damage on mitochondrial and lysosomal membranes through lipid peroxidation, which ultimately leads to cell death.

Conclusion

Results of this study suggest that MTBE decreases cell viability, which is associated with significant increase at intracellular reactive oxygen species (ROS) level and toxic alterations in mitochondria and lysosomes in human blood lymphocytes. These mentioned effects trigger depleted glutathione and lipid peroxidation. Moreover, MTBE showed its cytotoxic effects at low concentrations that may resemble to its concentrations in human blood following occupational and environmental exposure. It is therefore concluded that MTBE was capable of inducing oxidative stress and damage to mitochondria and lysosomes in lymphocytes at concentrations ranging from 5 to 40 μg/L, which may be present in human blood as a result of environmental and occupational exposure.

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