

# An investigation of methyl *tert*-butyl ether-induced cytotoxicity and protein profile in Chinese hamster ovary cells

GUANGSHAN XIE<sup>1-4\*</sup>, WEN-XU HONG<sup>1-3\*</sup>, LI ZHOU<sup>2</sup>, XIFEI YANG<sup>2</sup>, HAIYAN HUANG<sup>2</sup>,  
DESHENG WU<sup>2</sup>, XINFENG HUANG<sup>2</sup>, WEIGUO ZHU<sup>4</sup> and JIANJUN LIU<sup>1,2</sup>

<sup>1</sup>Shenzhen Research Institute of Population and Family Planning, Shenzhen, Guangdong 518040;

<sup>2</sup>Key Laboratory of Modern Toxicology of Shenzhen, Medical Key Laboratory of Guangdong Province, Medical Key Laboratory of Health Toxicology of Shenzhen, Shenzhen Center for Disease Control and Prevention, Shenzhen, Guangdong 518055; <sup>3</sup>Shenzhen Blood Center, Shenzhen, Guangdong 518035;

<sup>4</sup>Department of Chemistry, Xiangtan University, Xiangtan, Hunan 411105, P.R. China

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**Abstract.** Methyl *tert*-butyl ether (MTBE) is widely used as an oxygenating agent in gasoline to reduce harmful emissions. However, previous studies have demonstrated that MTBE is a cytotoxic substance that has harmful effects *in vivo* and *in vitro*. Although remarkable progress has been made in elucidating the mechanisms underlying the MTBE-induced reproductive toxicological effect in different cell lines, the precise mechanisms remain far from understood. The present study aimed to evaluate whether mammalian ovary cells were sensitive to MTBE exposure *in vitro* by assessing cell viability, lactate dehydrogenase (LDH) leakage, malondialdehyde (MDA) content and antioxidant enzyme activities. In addition, the effect of MTBE exposure on differential protein expression profiles was examined by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. MTBE exposure induced significant effects on cell viability, LDH leakage, plasma membrane damage and the activity of antioxidant enzymes. In the proteomic analysis, 24 proteins were demonstrated to be significantly affected by MTBE exposure. Functional analysis indicated that these proteins were involved in catalytic activity, binding, structural molecule activity, metabolic processes, cellular processes and localization, highlighting the fact that the cytotoxic mechanisms resulting from MTBE exposure are complex and diverse. The altered expression levels of two representative

proteins, heat shock protein family A (Hsp70) members 8 and 9, were further confirmed by western blot analysis. The results revealed that MTBE exposure affects protein expression in Chinese hamster ovary cells and that oxidative stress and altered protein levels constitute the mechanisms underlying MTBE-induced cytotoxicity. These findings provided novel insights into the biochemical mechanisms involved in MTBE-induced cytotoxicity in the reproductive system.

## Introduction

Methyl *tert*-butyl ether (MTBE) is a gasoline oxygenate additive that is used to increase the oxygen content of fuel, decreasing carbon monoxide and reducing air pollution (1). MTBE enters the environment during all phases of the fuel cycle (2). Due to its high volatility, high solubility and low biodegradation, MTBE moves through the soil and into the ground water more rapidly and remains there more persistently compared with other gasoline-related chemicals, resulting in groundwater contamination and global environmental pollution (3). The US Geological Survey reported that MTBE is one of the most frequently detected chemicals in shallow urban monitoring wells (4). High levels of MTBE are encountered during motor vehicle refueling, where attendants and customers are exposed to evaporated emissions (5,6). MTBE is readily absorbed when inhaled or ingested (7) with the metabolic pathways underlying this involving oxidative demethylation of MTBE to formaldehyde and *tert*-butanol by microsomal cytochrome P450 enzymes (8,9).

The effect of MTBE exposure on health has been assessed using a variety of study designs, including epidemiological investigations of large human populations and laboratory studies (3). Acute MTBE exposure causes a variety of symptoms, including headaches, and eye, nose and throat irritation (10). Previous studies in experimental rodent bioassays revealed that the incidence of three tumor types increased following chronic exposure to MTBE: Renal tubule cell tumors, testicular Leydig interstitial cell tumors and liver tumors (11-13). MTBE exposure also exerts toxic effects on cultured cell lines (14,15), affecting cell proliferation and cell transformation (16). DNA

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*Correspondence to:* Dr Wen-Xu Hong or Professor Jianjun Liu, Shenzhen Research Institute of Population and Family Planning, 4009 Xinzhou Road, Shenzhen, Guangdong 518040, P.R. China  
E-mail: wenxuhong@hotmail.com  
E-mail: bio-research@hotmail.com

\*Contributed equally

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damage in the form of fragmentation of the single and double helix has been reported in human lymphocytes exposed to MTBE (17). General stress is suspected to be the underlying reason behind the induction of toxicity (18). Increased MTBE-induced reactive oxygen species (ROS) production was also reported and revealed to be involved in its cytotoxicity in *in vitro* studies (14,19). These previous studies indicate that oxidative stress is one of the important mechanisms underlying MTBE-induced cytotoxicity.

Proteins are primary effectors of the response of biological systems to environmental alterations. Physiological or pathological conditions are reflected in protein expressions and enzyme activities (20). Proteomics is a high-throughput approach that has been extensively used to analyze adverse effects on cellular responses, discover novel biomarkers, and elucidate precise molecular mechanisms (21). Although remarkable progress had been made in previous studies regarding MTBE-induced reproductive toxicological effect *in vitro*, the precise mechanisms of MTBE-induced reproductive toxicology are far from understood. The present study aimed to evaluate whether mammalian ovary cells *in vitro* were sensitive to MTBE exposure, by measuring cell proliferation, lactate dehydrogenase (LDH) leakage, maleic dialdehyde (MDA) content and the enzymatic activity of antioxidant proteins. In addition, MTBE-induced effects on differential protein expression profiles were examined by two-dimensional electrophoresis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). All identified proteins were analyzed by bioinformatics and two representative proteins, heat shock protein family A (Hsp70) member 9 (Hspa9) and heat shock protein family A (Hsp70) member 8 (Hspa8), were confirmed by western blot analysis. The present study is the first, to the best of our knowledge, to investigate MTBE-induced effects on the proteome of cultured mammalian cells. These findings provided novel insights into the biochemical mechanisms involved in MTBE-induced cytotoxicity in the reproductive system.

## Materials and methods

**Reagents and chemicals.** MTBE (cat. no. 1634-04-4; 99.9% purity) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Chinese hamster ovary (CHO) cells were purchased from the cell bank at the Chinese Academy of Science (Shanghai, China). RPMI-1640 medium, fetal bovine serum (FBS), penicillin-streptomycin and trypsin were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit to measure cell viability was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Test kits for LDH, MDA, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). CyDye DIGE fluorescent dyes were obtained from GE Healthcare Life Sciences (Chalfont, UK). Hspa9 antibodies (cat. no. ab171089) were obtained from Abcam (Cambridge, UK). Hspa8 (cat. no. sc-7298) and  $\beta$ -actin (cat. no. sc-47778) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals were of analytical grade.

**Cell culture and proliferation assay.** CHO cells were cultured in RPMI-1640 media containing 10% FBS, 100  $\mu$ g/ml penicillin and 100  $\mu$ g/ml streptomycin in 5% CO<sub>2</sub> at 37°C. MTBE was administered at concentrations of 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM when cell confluence reached 80%, and the cells were treated for 6, 12 and 24 h. MTBE concentrations ranging between 0.5 and 100.0 mM were relatively high compared with expected environmental exposure (~0.45  $\mu$ M) (19,22). However, 100 mM MTBE is a lower concentration compared with that used in previous studies focusing on MTBE toxicity (210 mM) (23).

CHO cell viability was assessed using an MTT assay, as described previously with minor modifications (24). Briefly, cells (100  $\mu$ l) were seeded into 96-well culture plates (Corning Incorporated, Corning, NY, USA) at a density of  $8.0 \times 10^3$  cells/well, and the cells were treated with MTBE at the stated concentrations and incubated at 37°C in an atmosphere of 5% CO<sub>2</sub> for 6, 12 and 24 h. Following treatment, 20  $\mu$ l MTT working solution (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 h. The MTT solution was discarded and 150  $\mu$ l dimethyl sulfoxide was added to each well. The optical density was measured on a microplate reader at 490 nm. Exposure to MTBE to 12 h resulted in the greatest inhibition of cell viability, and was subsequently used for further biochemical analysis.

**LDH leakage assay.** The fraction of total LDH activity in the cell supernatants was used as an indicator of membrane leakage or cell lysis. Cytosolic LDH activity was estimated, as previously described (25). Following 12 h exposure to MTBE at 37°C in a 5% CO<sub>2</sub> incubator, the medium was collected and the level of LDH released by cells was determined using an assay kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol. Following the reaction, the absorbance of each sample was measured at a wavelength of 450 nm with an Infinite M1000 Microplate Reader (TECAN Group, Ltd., Männedorf, Switzerland).

**Lipid peroxidation assay.** Lipid peroxidation was assessed by determining the levels of thiobarbituric acid reactive substances, using the method previously described by Mihara and Uchiyama (26). CHO cells were seeded into 6-well plates ( $1.0 \times 10^6$  per well) and were allowed to attach for 24 h. The medium was replaced with RPMI-1640 medium along with 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE for 12 h. The treated cells were fragmented and centrifuged at  $4,000 \times g$  at 37°C for 10 min, and the supernatants were measured using the MDA test kit (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. The absorbance was determined using an Infinite M1000 Microplate Reader (TECAN Group, Ltd.) at wavelength of 532 nm. MDA content was calculated, according to a standard curve and expressed as nmol/mg protein. The protein content was determined using a Bradford assay, with FBS used as the standard (27).

**Enzyme activity assays of SOD, CAT and GSH-Px.** The enzyme activity of SOD, CAT and glutathione GSH-Px were examined to investigate the effect of MTBE-induced oxidative stress. These indexes were detected with corresponding assay kits (Nanjing Jiancheng Bioengineering Institute), according

to the manufacturer's protocol. CHO cells were seeded into 6-well plates ( $1.0 \times 10^6$  per well) and were allowed to attach for 24 h. The medium was replaced with RPMI-1640 medium along with 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE for 12 h. The medium was subsequently removed and the cells were pooled in a 1.5 ml tube. The cells were centrifuged at  $3,000 \times g$  at  $4^\circ\text{C}$  for 10 min, and the pellet was resuspended with  $1,000 \mu\text{l}$  PBS and centrifuged at  $10,000 \times g$  at  $4^\circ\text{C}$  for 15 min. The supernatant was collected for use in SOD, CAT and GSH-Px assays. The activities of SOD, CAT and GSH-Px were calculated according to a standard curve and expressed as U/mg protein. Protein content was determined using the Bradford assay with FBS as the standard (27).

*Protein labeling and two-dimensional difference gel electrophoresis (2-D DIGE).* Treated and untreated samples were minimally labeled ( $25 \mu\text{g}$  proteins per 200 pmol dyes) with cyanine (Cy)2, Cy3 or Cy5 fluorescent dyes, according to the manufacturer's protocol (GE Healthcare Life Sciences). A reference design was used, in which a 50.0 mM MTBE exposure condition and control was labeled once with Cy3 and once with Cy5, with the reference sample labeled with Cy2 to generate an internal standard for normalization. By including the internal standard on each gel, the abundance of each protein spot of the individual biological samples was measured relative to its corresponding spot in the internal standard present on the same gel.

For 2-D DIGE, immobilized pH gradient (IPG) strips were dehydrated in an Ettan IPGphor 3 isoelectric focusing system (GE Healthcare Life Sciences). Later samples were focused until a total of 8,000 Vh was achieved. Each strip was embedded on top of sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and separated on an Ettan DALT electrophoresis system (GE Healthcare Life Sciences). A loading of 1,000 mg unlabeled proteins was performed in parallel for spots picking and in-gel digestion.

*Image analysis.* Following 2-D DIGE, the gels were scanned using a Typhoon Trio Variable Mode Imager (GE Healthcare Life Sciences) at excitation/emission wavelengths of 532(blue)/580, 532(green)/425 and 633(red)/425 nm for Cy2, Cy3 and Cy5, respectively. The resultant maps were analyzed using Decyder 2D v6.5 (GE Healthcare Life Sciences). Gel images were imported into the software, and the protein spots were analyzed with the differential in-gel analysis and biological analysis modules. To compare protein expression levels, the spot volume of the Cy3 and Cy5 samples was normalized to the corresponding spot volume of the Cy2 internal standard samples from the same gel. Student's t-test and one-way analysis of variance (ANOVA) were used to calculate the statistical significance. The spots exhibiting statistically significant differences ( $P < 0.05$ ) were further analyzed.

*Protein identification and bioinformatics analysis.* The spots of interest detected by Decyder software analysis were manually excised from the 2-D DIGE gel and stained using Coomassie Brilliant Blue G-250 staining solution (0.12% Coomassie Brilliant Blue G-250, 20% ethanol, 10% phosphoric acid and 10% ammonia sulfate). The protein spots were washed

three times with Milli-Q Ultrapure water (Merck Millipore, Darmstadt, Germany) and were subsequently destained with destaining solution (25 mM ammonium bicarbonate/50% acetonitrile). Gel pieces were dehydrated with 100% acetonitrile and incubated at  $37^\circ\text{C}$  for 30 min. Each gel piece was digested overnight at  $37^\circ\text{C}$  with  $0.03 \mu\text{g}$  sequencing-grade trypsin (Promega, Madison, WI, USA) in 15 ml of 25 mM ammonium bicarbonate buffer. The tryptic peptides were used for MALDI-TOF-MS/MS analysis.

All mass spectrums were acquired on an AutoFlex MALDI-TOF/TOF with LIFT technology (Bruker Corporation, Billerica, MA, USA). Protein identification by PMF and MS/MS spectra was performed using the MASCOT search engine 2.2 (Matrix Science, Inc., Boston, MA, USA) and BioTools software (version 3.2 SR4; Bruker Corporation). Search parameters were set as follows: Taxonomy: *Mus musculus*; enzyme: Trypsin; missed cleavage: 1; fixed modification: Carbamidomethyl (C); variable modification: Oxidation (M); peptide tolerance:  $\pm 100$  ppm; mass tolerance:  $\pm 0.3$  kDa; ions: [M+H]. Confident matches in the SwissProt database ([www.ebi.ac.uk/uniprot](http://www.ebi.ac.uk/uniprot)) were defined by the MASCOT score, the sequence coverage by matching peptides and statistical significance ( $P < 0.05$ ). Protein ontology classification was performed by importing proteins into the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system (<http://www.pantherdb.org/>) (28). The proteins were grouped according to the association with molecular functions and biological processes.

*Western blot analysis.* The MTBE-induced effects on Hspa9 and Hspa8 protein expression levels were confirmed by western blot analysis. CHO cells underwent 12 h exposure to 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE and were lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and collected by scraping. Protein concentration was determined using the Bradford assay, with FBS used as the standard. The resultant whole lysates ( $20 \mu\text{g}/\text{lane}$ ) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrically transferred onto a polyvinylidene fluoride membrane (GE Healthcare Life Sciences). The membranes were blocked in 5% milk dissolved in Tris-buffered saline with 0.04% Tween-20 (TBST), and were subsequently incubated with Hspa9 (1:1,000), Hspa8 (1:1,000) and  $\beta$ -actin antibodies (1:2,000) at  $4^\circ\text{C}$  overnight. Following primary antibody incubation, the membranes were washed with TBST buffer three times and incubated at room temperature for 2 h with goat anti-mouse IgG-HRP (cat. no. sc-2031) or goat anti-rabbit IgG-HRP (cat. no. sc-2054) secondary antibodies at 1:4,000 dilutions (Santa Cruz Biotechnology, Inc.). The blots were incubated in Super signal<sup>®</sup>West Dura Extended Duration Substrate (Thermo Fisher Scientific, Inc.) and detected using the ECL<sup>™</sup> western blotting detection system (Image-Quant<sup>™</sup> RT; GE Healthcare Life Sciences).

*Statistical analysis.* All experiments were replicated  $\geq 3$  independent times and the data are expressed as the mean  $\pm$  standard deviation. The data were analyzed using one-way ANOVA tests followed by Dunnett's tests to determine the statistical difference between treatment groups.

These tests were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cytotoxic effect of MTBE exposure in CHO cells.** To study the cytotoxic effect of MTBE exposure in CHO cells, cell viability was assessed using an MTT assay following treatment with 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE for different durations: 6, 12 and 24 h. Incubation of CHO cells for 6 h with 50.0 and 100.0 mM of MTBE resulted in a significant decrease in cell viability compared with the control cells ( $P = 0.0086$  and  $P = 0.001$ , respectively; Fig. 1). When CHO cells were incubated with MTBE for 12 h cell viability significantly decreased at concentrations  $\geq 5.0$  mM compared with control cells (5.0 mM,  $P = 0.036$ ; 25.0 mM,  $P = 0.042$ ; 50.0 mM,  $P = 0.023$ ; 100.0 mM,  $P = 0.026$ ; Fig. 1). When CHO cells were incubated with MTBE for 24 h, cell viability significantly decreased at 50.0 and 100.0 mM compared with the control cells ( $P = 0.028$  and  $P = 0.025$ , respectively; Fig. 1). By comparing cell viability rates at the different exposure times, 12 h MTBE exposure was selected for further analysis of MTBE-induced cytotoxicity and oxidative stress in CHO cells as it resulted in the highest inhibition of cell viability. The concentration of 50.0 mM was selected for 2DE analysis, and 5.0 and 50.0 mM were selected for western blot analysis.

**MTBE exposure leads to plasma membrane damage in CHO cells.** Plasma membrane damage was assessed by monitoring LDH release following 12 h exposure to 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE. MTBE exposure induced a significant increase in LDH leakage at concentrations  $\geq 25.0$  mM compared with the control group (25.0 mM,  $P = 0.001$ ; 50.0 mM,  $P = 0.002$ ; 100.0 mM,  $P = 0.042$ ; Fig. 2).

**MTBE exposure induces oxidative stress in CHO cells.** The effect of 0, 0.5, 5.0, 25.0, 50.0 or 100.0 mM MTBE on lipid peroxidation, as indicated by MDA content, was estimated. MDA is one of the end products of lipid peroxidation and the MDA assay has previously been used for assessing oxidative damage (29). Exposure to MTBE for 12 h induced a significant increase in MDA levels in CHO cells, at all concentrations  $\geq 5.0$  mM, compared with control cells (5.0 mM,  $P < 0.001$ ; 25.0 mM,  $P < 0.001$ ; 50.0 mM,  $P < 0.001$ ; 100.0 mM,  $P < 0.001$ ; Fig. 3), indicating an increase in lipid peroxidation.

SOD, CAT and GSH-Px are three important antioxidative enzymes, and their activity levels following MTBE exposure were assessed. Exposure to 50.0 mM or 100.0 mM MTBE for 12 h induced a significant increase of SOD activity compared with the control cells ( $P = 0.035$  and  $P = 0.002$ , respectively; Fig. 4). Exposure to 25.0, 50.0 or 100.0 mM MTBE induced a significant increase of CAT activity in CHO cells compared with control cells ( $P < 0.001$ ,  $P < 0.001$  and  $P < 0.001$ , respectively; Fig. 5) and a significant increase of GSH-Px activity compared with control cells ( $P < 0.001$ ,  $P < 0.001$  and  $P < 0.001$ , respectively; Fig. 6).

**Effect of MTBE exposure on the CHO cell proteome.** To assess the effect of MTBE exposure on protein profiles, a quantitative

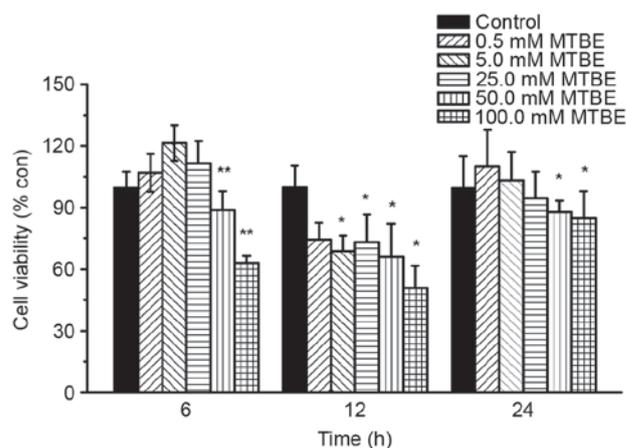


Figure 1. Effect of MTBE on Chinese hamster ovary cell viability. Cell viability was assessed at 6, 12 and 24 h following various treatments with MTBE. The data are presented as the mean  $\pm$  standard deviation (\* $P < 0.05$  and \*\* $P < 0.01$  vs. control). MTBE, methyl *tert*-butyl ether; con, control.

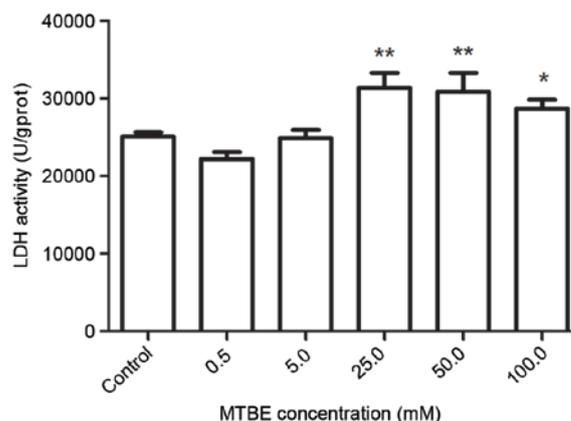


Figure 2. Effect of MTBE on LDH activity in Chinese hamster ovary cells. The LDH activity was assessed following treatment with various concentrations of MTBE. The data are presented as the mean  $\pm$  standard deviation (\* $P < 0.05$  and \*\* $P < 0.01$  vs. the control). MTBE, methyl *tert*-butyl ether; LDH, lactate dehydrogenase.

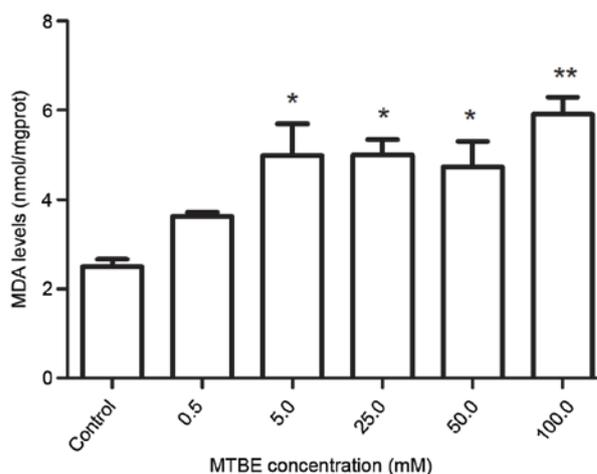


Figure 3. Effect of MTBE on lipid peroxidation in Chinese hamster ovary cells. Following 12 h exposure to various concentrations of MTBE, the MDA levels were assessed. The data are presented as the mean  $\pm$  standard deviation (\* $P < 0.05$  and \*\* $P < 0.01$  vs. control). MTBE, methyl *tert*-butyl ether; MDA, maleic dialdehyde.

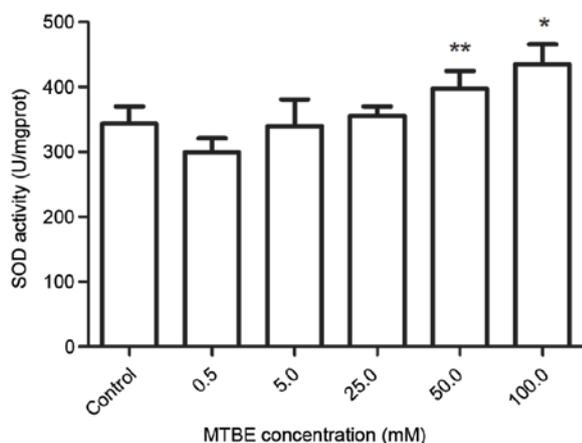


Figure 4. Effect of MTBE on SOD activity in Chinese hamster ovary cells. Following 12 h exposure to various concentrations of MTBE, the SOD activity was assessed. The data are presented as the mean  $\pm$  standard deviation (\* $P$ <0.05 and \*\* $P$ <0.01 vs. the control). MTBE, methyl *tert*-butyl ether; SOD, superoxide dismutase.

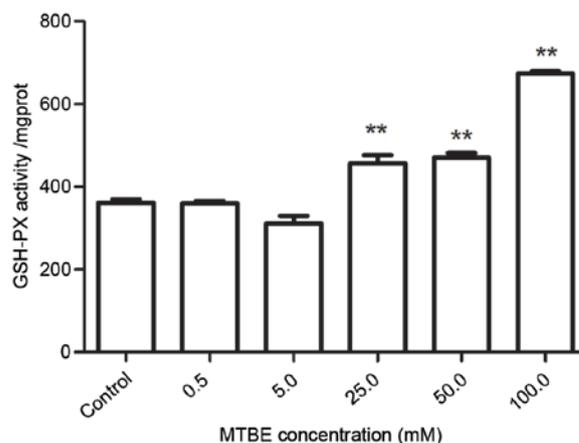


Figure 6. Effect of MTBE on GSH-Px activity in Chinese hamster ovary cells. Following 12 h exposure to various concentrations of MTBE, the GSH-Px activity was assessed. The data are presented as the mean  $\pm$  standard deviation (\*\* $P$ <0.01 vs. control). MTBE, methyl *tert*-butyl ether; GSH-Px, glutathione peroxidase.

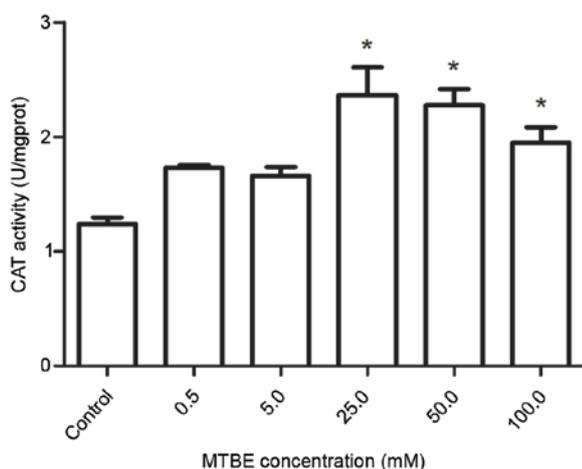


Figure 5. Effect of MTBE on CAT activity in Chinese hamster ovary cells. Following 12 h exposure to various concentrations of MTBE, the CAT activity was assessed. The data are presented as the mean  $\pm$  standard deviation (\* $P$ <0.05 vs. control). MTBE, methyl *tert*-butyl ether; CAT, catalase.

proteomic analysis by 2DE was performed between 50.0 mM MTBE-treated CHO cells and the untreated control cells. Comparative analysis of the protein profiles was performed between the four test groups using DeCyder software (version 6.5; Fig. 7). The matching of protein spots was corrected by inserting landmarks. With this labeling strategy, it is possible to analyze all biological replicates in a single experimental run. Following 2-D DIGE analysis, protein spots demonstrating  $\geq 1.2$ -fold differential expression were selected for MS/MS identification (30).

A total of 27 protein spots demonstrated statistically significant differences between the 50.0 mM MTBE-exposed CHO cells and untreated control cells, with 19 spots were downregulated and 8 spots were upregulated in 50.0 mM MTBE-exposed CHO cells compared with the control cells ( $P$ <0.05; Table I; Fig. 8). The spots were digested with trypsin and MALDI-TOF/TOF mass spectrometry was applied to obtain high-resolution peptide mass fingerprints and peptide

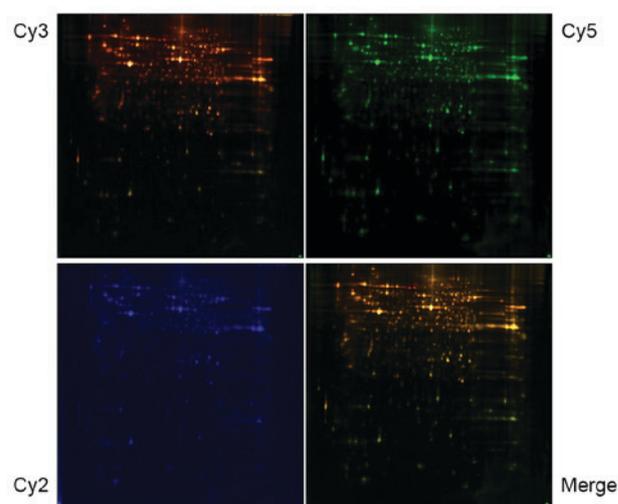


Figure 7. Representative two-dimensional difference gel electrophoresis (DIGE) images. Samples treated with 50.0 mM methyl *tert*-butyl ether or control samples were labeled with Cy3 and Cy5, respectively, while the protein reference pool (internal standard) was labeled with Cy2. The images were scanned at excitation/emission wave length, 488/520 (Cy2), 532/580 (Cy3), and 633/670 (Cy5) nm. The green spots represent upregulated proteins, while the red spots represent downregulated proteins in treated groups. Cy, cyanine.

fragment fingerprints. The proteins were subsequently identified by searching the SwissProt database. Of the upregulated spots, 2 were identified as ATP synthase subunit  $\beta$  (Atp5b; Table I), 2 were identified as heat shock protein family A (Hsp70) member 8 (Hspa8; Table I) and 2 were identified as  $\beta$ -actin (Actb; Table I).

**Functional categories of identified proteins.** The 24 identified proteins were classified with respect to their molecular functions (Fig. 9A) and biological processes (Fig. 9B), according to PANTHER (28). The most dominant function that the identified proteins were involved in was catalytic activity (32.1%), followed by binding (28.6%) and structural molecule activity (17.9%; Fig. 9A). The most dominant biological processes

Table 1. Differentially expressed proteins of membrane fractions from CHO cells following MTBE exposure.

Entry	Protein name	Gene name	Accession no.	Score	Mw/PI	AV ratio	P-value <sup>a</sup>
1	40S ribosomal protein SA	<i>Rpsa</i>	RSSA_MOUSE	254	32,931/4.80	2.02	0.03000
2	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, beta polypeptide	<i>Atp5b</i>	ATPB_MOUSE	878	56,265/5.19	1.80	0.04400
	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, beta polypeptide	<i>Atp5b</i>	ATPB_MOUSE	407	56,265/5.19	1.72	0.01500
3	Heat shock protein family A member 9	<i>Hspa9</i>	GRP75_MOUSE	88	73,768/5.91	1.77	0.04700
4	Eukaryotic translation elongation factor 1 delta	<i>Eef1d</i>	EF1D_MOUSE	107	31,388/4.91	1.70	0.04400
5	Heat shock protein family A (Hsp70) member 8	<i>Hspa8</i>	HSP7C_MOUSE	100	71,055/5.37	1.61	0.01100
	Heat shock protein family A (Hsp70) member 8	<i>Hspa8</i>	HSP7C_MOUSE	171	71,055/5.37	1.52	0.04400
6	Beta-actin n, cytoplasmic	<i>Actb</i>	ACTB_MOUSE	103	42,052/5.29	1.52	0.04400
	Beta-actin, cytoplasmic	<i>Actb</i>	ACTB_MOUSE	384	42,052/5.29	1.25	0.02300
7	Beta-actin -like protein 2	<i>Actbl2</i>	ACTBL_MOUSE	53	42,319/5.30	1.52	0.04400
8	Heterogeneous nuclear ribonucleoprotein K	<i>Hnrnpk</i>	HNRPK_MOUSE	261	51,230/5.39	1.48	0.01200
9	T-complex protein 1 subunit $\alpha$	<i>Tcp1</i>	TCPA_MOUSE	72	60,867/5.82	1.31	0.03900
10	Ubiquinol-cytochrome c reductase core protein I	<i>Uqcrc1</i>	QCRI_MOUSE	189	53,446/5.81	1.29	0.03500
11	FK506 binding protein 4	<i>Fkbp4</i>	FKBP4_MOUSE	133	51,939/5.54	1.27	0.00210
12	Pyruvate kinase, muscle, isozymes M1/M2	<i>Pkm</i>	KPYM_MOUSE	31	58,378/7.18	1.26	0.04400
13	Heat shock protein family D (Hsp60) member 1	<i>Hspd1</i>	CH60_MOUSE	121	61,088/5.91	1.25	0.03600
14	Enolase 1	<i>Eno1</i>	ENOA_MOUSE	206	47,453/6.37	1.22	0.00740
15	Eukaryotic translation initiation factor 4E	<i>Eif4e</i>	IF4E_MOUSE	201	25,266/5.79	1.22	0.03900
16	Protein disulfide isomerase family A member 3	<i>Pdia3</i>	PDIA3_MOUSE	207	57,099/5.88	1.21	0.04800
17	Actin, $\alpha 2$ , smooth muscle, aorta	<i>Acta2</i>	ACTA_MOUSE	117	42,381/5.23	-7.30	0.02300
18	Peptidylprolyl isomerase A	<i>Ppia</i>	PPIA_MOUSE	91	18,131/7.74	-2.18	0.00073
19	Calponin 3	<i>Cnn3</i>	CNN3_MOUSE	142	36,577/5.46	-1.29	0.02700
20	ENY2, transcription and export complex 2 subunit	<i>Eny2</i>	ENY2_MOUSE	18	11,635/9.39	-1.26	0.03700
21	ATP synthase, H <sup>+</sup> transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	<i>Atp5a1</i>	ATPA_MOUSE	627	59,830/9.22	-1.25	0.02200
22	Histone cluster 1 H2B family member B	<i>Hist1h2bb</i>	H2B1B_MOUSE	96	13,944/10.31	-1.23	0.00047
23	Receptor for activated C kinase 1	<i>Gnb2l1</i>	GBLP_MOUSE	31	35,511/7.60	-1.22	0.03600
24	Nudix hydrolase 17	<i>Nudt17</i>	NUD17_MOUSE	29	33,116/5.44	-1.21	0.01700

<sup>a</sup>P-value was obtained by t-test. Mw, molecular weight; PI, isoelectric point; AV ratio, average ratio.

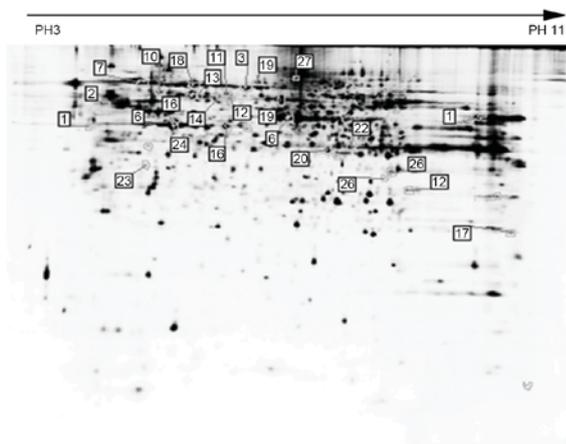


Figure 8. Differentially expressed protein spots identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry. The differentially expressed proteins were marked on the two-dimensional gels and numbered.

included metabolic processes (30.2%), cell processes (18.9%) and localization (15.1%).

*Confirmation of differential expression levels of GRP7 and Hspa8 in MTBE-induced CHO cells.* Hspa9 and Hspa8 were selected as representative proteins and subjected to western blot analysis to confirm the differential expression levels (Fig. 10A). The protein expression levels of Hspa9 and Hspa8 were significantly upregulated in CHO cells following 12 h MTBE exposure (50.0. mM) compared with the control cells ( $P < 0.01$  and  $P < 0.01$ , respectively, Fig. 10B and C, respectively). These results were consistent with the 2DE data (Table I).

## Discussion

MTBE is primarily used as an oxygenating agent in gasoline to reduce harmful emissions. Occupational MTBE exposure affects all workers exposed to gasoline, and environmental MTBE exposure (in the air and groundwater) affects the general population (31,32). Although available data demonstrate conflicting results, there is evidence that MTBE is a toxic substance that has harmful effects *in vivo* and *in vitro*, with MTBE exposure reported to induce reproductive toxicity in multiple cell lines (14,15,33-35). *In vitro* cell systems are an excellent model for studying the molecular mechanisms of toxicity since they allow rapid and reliable results that can be easily confirmed without using laboratory animals. Numerous measurement endpoints for cytotoxicity have been established and the results were used to assess the basal cytotoxicity (36,37). Consistent results from previous *in vitro* studies led to higher tier *in vivo* tests and, eventually, to hazard identification and classification. In the present study, MTBE-induced effects on cytotoxicity and oxidative stress were investigated in CHO cells; an ovary cell line widely used for assessing chemical-induced cytotoxicity. Furthermore, the protein profile of MTBE-exposed and non-exposed CHO cells was analyzed using a proteomic approach, providing data at a molecular level, which is useful for more informative risk assessment and mechanism studies.

MTBE significantly inhibited CHO cell viability following 6, 12 and 24 h exposure, with the cell viability decreasing as result of cytotoxicity. In the present study, the concentration of MTBE ranged between 0.50 and 100.0 mM, with 100.0 mM being lower compared with the 210.0 mM used in previous studies on MTBE toxicity (23). By comparing cell viability following 6, 12 and 24 h exposure, 12 h MTBE exposure was selected for further analysis as it induced the highest inhibition of cell viability (Fig. 1). It is hypothesized that 12 h produced greater inhibition as MTBE is highly volatile and the concentration of MTBE is decreased in a time-dependent manner. LDH is a stable cytoplasmic enzyme that is present in all cells, and it is rapidly released into the cell culture media when the plasma membrane is damaged. Increased LDH release in CHO cells revealed that MTBE exposure alters cell membrane properties (Fig. 2) (25). Increased plasma membrane damage may directly induce cell necrosis, resulting in the significantly increased ratio of cell necrosis and apoptosis demonstrated at higher concentrations. The data obtained in the present study indicated that MTBE exposure has a direct cytotoxic effect, decreasing cell viability and inducing plasma membrane damage in CHO cells.

Oxidative stress is considered to be an important mechanism underlying MTBE-induced reproductive toxicity (14,19,38). The extent of membrane lipid peroxidation was estimated by measuring MDA formation (39), which is a byproduct of lipid peroxidation considered to be a biomarker of cellular damage (40). MTBE exposure significantly increased the MDA content, implying an increased peroxide level in CHO cells. In addition, MTBE exposure induced significant increases in antioxidant enzyme activities, including SOD, CAT and GSH-Px. The apparent reverse concentration-response association observed in CAT activity levels at higher MTBE concentrations may be due to the low solubility and high volatility of MTBE. The above results demonstrated that MTBE exposure exerted oxidative stress in CHO cells, which may be the underlying mechanism of MTBE-induced cytotoxicity. Cellular oxidative status is determined by the balance between the production of ROS and their destruction by various antioxidant enzymes. In particular, ROS are scavenged continuously by antioxidative enzymes, including SOD, CAT and GSH-Px (41). SOD is an important antioxidant that eliminates excess cellular oxidants. CAT is a key antioxidant enzyme in the body's defense against oxidative stress, converting hydrogen peroxide to water and oxygen and thereby mitigating the cytotoxic effects of hydrogen peroxide (42). GSH-Px also functions in the detoxification of hydrogen peroxide, and provides an effective protection mechanism against cytosolic injury by eliminating  $H_2O_2$  and lipid peroxides (43). Therefore, by measuring the levels of these antioxidative enzymes, the status of oxidative stress in cells can be indirectly indicated. ROS production was observed to decrease following MTBE exposure (data not shown), which may be trapped and scavenged by endogenous antioxidants including SOD, CAT and GSH-Px, which may be overexpressed in response to MTBE exposure. The enhanced MDA levels and increased activities of SOD, CAT and GSH-Px indicated that increased oxidative stress contributes to the cytotoxicity of MTBE.

High-throughput techniques, including proteomics, provide effective strategies for toxicological studies and are regarded

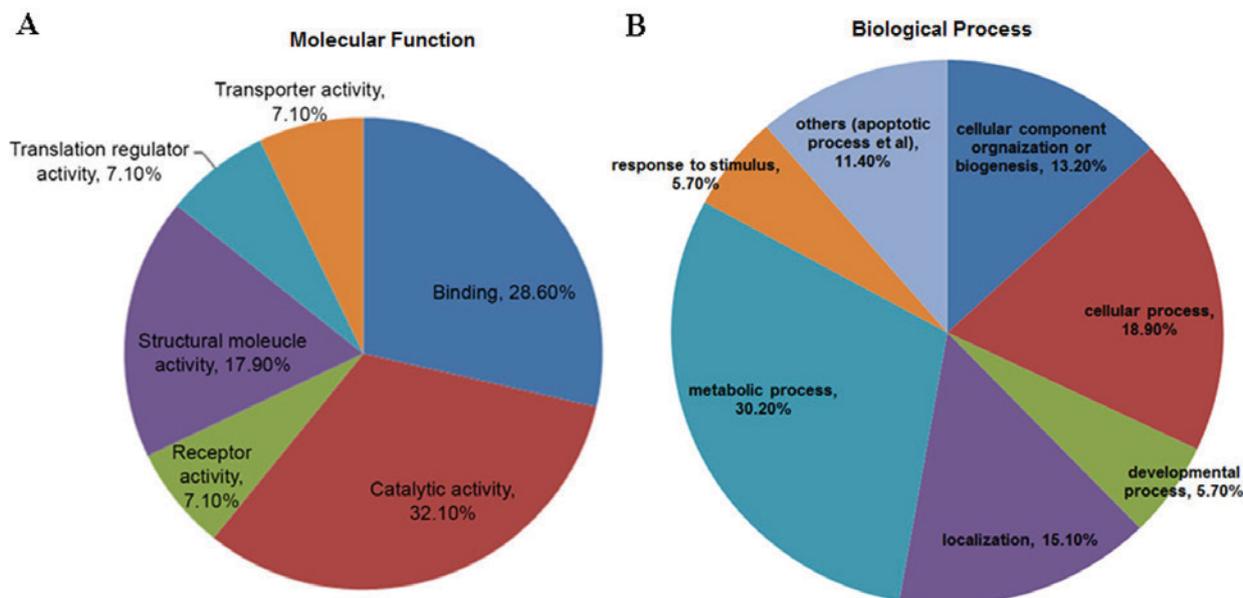


Figure 9. Functional classifications of the identified proteins according to (A) molecular function and (B) biological processes, as determined by Protein Analysis Through Evolutionary Relationships.

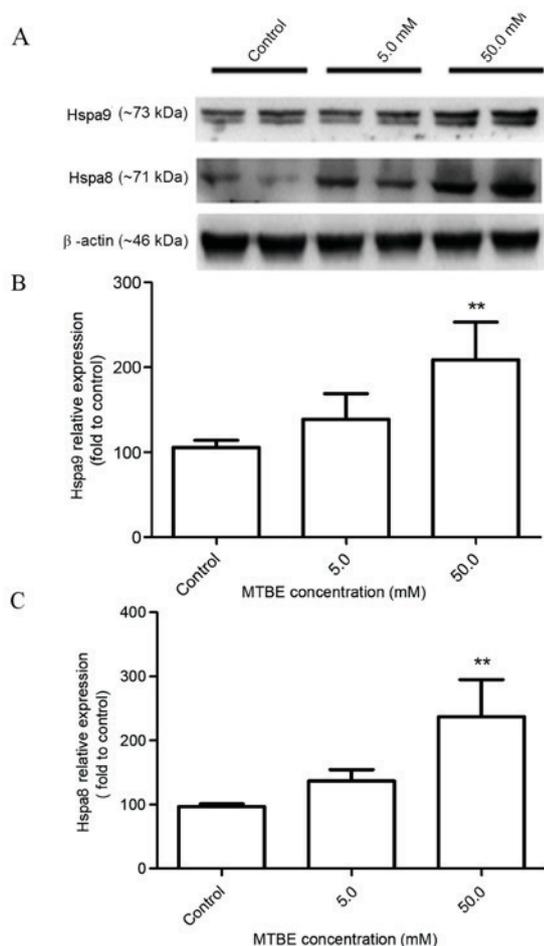


Figure 10. Expression levels of Hspa proteins following treatment with MTBE. (A) Differential expression of Hspa9 and Hspa8 following 12 h exposure to MTBE, as confirmed by western blot analysis.  $\beta$ -actin was included as an internal control. The protein expression of (B) Hspa9 and (C) Hspa8 was quantified, relative to  $\beta$ -actin. The data are presented as the mean  $\pm$  standard deviation ( $n=3$ ; \*\* $P<0.01$  vs. control). Hspa, heat shock protein family A (Hsp70); MTBE, methyl *tert*-butyl ether.

as a powerful tool to investigate cellular responses to environmental and occupational pollutants (44). In the present study, 2-D DIGE technology, in combination with mass spectrometry, was applied to detect differentially expressed proteins following MTBE exposure. Differentially expressed proteins were subsequently analyzed by bioinformatics and confirmed by western blot analysis. As a result, 24 proteins associated with MTBE-induced toxicity were identified. Of these, 16 were upregulated and 8 were downregulated in CHO cells following MTBE exposure. Using bioinformatics analysis (28), the identified proteins were classified into different groups based on their molecular functions and biological processes. The most dominant function of the identified protein was the catalytic activity, followed by binding and structural molecule activity. The most dominant biological processes included metabolic processes, cellular process and localization. These results demonstrated the complexity of the mechanisms underlying MTBE cytotoxicity in CHO cells.

Cellular stress, including oxidative stress, activates the nuclear factor, erythroid 2 like 2 (Nrf2) pathway. Under oxidative stress Nrf2 dissociates from kelch-like ECH associated protein 1 and translocates into the nucleus, activating target genes by heterodimerizing with small MAF transcription factors and binding to antioxidant-responsive elements (45–47). Activation of both Nrf2 and transcription factors coordinates and regulates the convergence of oxidative stress signaling (48). In the present study, transcription factors, including enhancer of yellow 2 transcription factor homolog were identified as being differentially expressed following MTBE exposure. The stress-activated mitogen activated protein kinase (MAPK) pathway, which involves c-Jun NH2-terminal kinase, p38 and MAPK2, is highly conserved and transduces cellular information in response to a variety of distinct signals (49). Cells experience an accumulation of misfolded and aggregated proteins when exposed to stress. Protective mechanisms involve the rapid induction of heat

shock protein (HSP) expression, and activation of MAPK signaling pathways. Together, the activities directed by HSPs and MAPKs influence cellular responses to oxidative stress. Several previous studies have demonstrated the direct inhibition of pro-apoptotic stress signaling and promotion of pro-survival pathways by HSPs (50,51). Therefore, the present study focused on HSPs, selecting two for confirmation by western blot analysis.

Hspa9 is a highly conserved member of the heat shock cognate protein family and is observed in multiple subcellular sites (52). It is involved in multiple physiological functions, including mitochondrial import, antigen processing, and the control of cell proliferation and differentiation (53). Hspa9 overexpression was previously demonstrated to significantly inhibit H<sub>2</sub>O<sub>2</sub> exposure-induced ROS accumulation in liver cells (54). In the present study, MTBE exposure was revealed to upregulate the expression of Hspa9 in both proteomic and western blot analysis. Hspa8 is constitutively expressed and is involved in housekeeping functions associated with chaperone-mediated autophagy and prevention from protein aggregation under stress conditions (55), and overexpression of Hspa8 is also known to be associated with necrosis (56). The majority of cells possess intrinsic defense mechanisms that protect against oxidative stress/injury. Hspa8 has been demonstrated to be involved in the signal transduction and apoptosis process, and translocates to the nucleus following exposure to heat shock (57,58). Upregulation of Hspa8 in leukemic cells was demonstrated to contribute to cell cycle disruption and lead to cell proliferation (59,60). Hspa8 was also revealed to contribute to the regulation of cancer cell growth by secretion in the extracellular environment (61). Abnormal Hspa8 expression has also been observed during stress responses following exposure to linear alkylbenzene sulfonate and other chemical pollutants (62,63). In the present study, upregulation of Hspa8 and Hspa9 revealed that MTBE exposure induced affected HSP expression. When taken together, the results of the present study, including the increase in MDA content and SOD, CAT and GSH-Px activity, suggested that a redox imbalance occurs in CHO cells following MTBE exposure.

In conclusion, the primary aim of the present study was to examine cytotoxicity and protein expression alteration in CHO cells following MTBE exposure. MTBE exposure induced significant effects on cell viability, LDH leakage, plasma membrane damage and the activities of antioxidant enzymes. In the proteomic analysis, 24 proteins were demonstrated to be significantly modulated by MTBE exposure. Functional analysis indicated that these proteins were involved in catalytic activity, binding, structural molecule activity, metabolic and cellular processes, and localization, highlighting the fact that the cytotoxic mechanisms of MTBE are complex and diverse. The altered expression of two representative proteins, Hspa9 and Hspa8, was further confirmed by western blot analysis. These results revealed the effect of MTBE exposure on protein expression in CHO cells, and the data indicated that oxidative stress and altered protein expression constituted the mechanisms underlying MTBE-induced cytotoxicity. Therefore, the present study provided useful scientific information and enhanced the understanding of the molecular mechanisms underlying MTBE-induced oxidative stress and reproductive toxicity

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