Genotoxicity of Motorcycle Exhaust Particles \textit{In Vivo} and \textit{In Vitro}

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We studied the genotoxic potency of motorcycle exhaust particles (MEP) by using a bacterial reversion assay and chromosome aberration and micronucleus tests. In the bacterial reversion assay (Ames test), MEP concentration-dependently increased TA98, TA100, and TA102 revertants in the presence of metabolic-activating enzymes. In the chromosome aberration test, MEP concentration-dependently increased abnormal structural chromosomes in CHO-K1 cells both with and without S9. Pretreatment with antioxidants (α-tocopherol, ascorbate, catalase, and NAC) showed varying degrees of inhibitory effect on the MEP-induced mutagenic effect and chromosome structural abnormalities. In the \textit{in vivo} micronucleus test, MEP dose-dependently induced micronucleus formation in peripheral red blood cells after 24 and 48 h of treatment. The increase of micronucleated reticulocytes induced by MEP was inhibited by pretreatment with α-tocopherol and ascorbate. The fluorescence intensity of DCFH-DA–loaded CHO-K1 cells was increased upon the addition of MEP. Our data suggest that MEP can induce genotoxicity through a reactive oxygen species–(ROS–) dependent pathway, which can be augmented by metabolic activation. Alpha-tocopherol, ascorbate, catalase, and NAC can inhibit MEP-induced genotoxicity, indicating that ROS might be involved in this effect.

Key Words: motorcycle exhaust particles; Ames test; reactive oxygen species; chromosome aberration; micronucleus; genotoxicity.

Recent prospective cohort studies have shown that long-term exposures to particulate air pollution might be associated with increases in the rates of morbidity and mortality from respiratory and cardiovascular diseases in the general population (Abbey et al., 1995; Dockery et al., 1993; Pope et al., 1995). In addition to systemic toxicity, the possible genotoxicity of small particulate matter has been investigated in recent years. On metabolism, activation, or accumulation, pollutants can become extremely toxic to vital organs, and this is often related to a strong genotoxic effect (Baulig et al., 2003; Harris et al., 1978). Airborne particles have been shown to induce chromosome aberrations (Seemayer et al., 1994), sister chromatig exchanges (Hornberg et al., 1996), the formation of DNA adduct (Gallagher et al., 1990), tumorigenicity (Heussen et al., 1996), and embroyotoxicity (Matsumoto and Kashimoto, 1986). In some industrial cities, the exhaust of diesel vehicles is considered a major source of air pollution that is "probably carcinogenic" (Aoki et al., 2001; Baeza Squiban et al., 1999). Diesel exhaust particles (DEP) are composed of carbon nuclei, absorbed organic compounds, and trace heavy metals including iron and copper (Schuetzle and Lewtas, 1986; Schuetzle et al., 1981). The absorbed organic compounds consist of some highly mutagenic chemicals including polycyclic aromatic hydrocarbons (PAHs) and nitroaromatic hydrocarbons (Grimmer et al., 1987; Lewtas, 1988) and were shown to cause pulmonary tumors (Mauderly et al., 1987).

In Taiwan, motorcycles are widely used; more than 11 million motorcycles were registered in 2000 (Ministry of Transportation and Communications, 2000). The use of motorcycles, especially those with two-stroke engines, introduce about 16,000 and 15,000 tons of total suspended particles and particulate matter of 10 μm (PM10), respectively, per year in Taiwan (Environment Protection Agency, 2000). However, the impact of motorcycle exhaust on the environment and its biological effects are relatively unknown. Motorcycle exhaust particles (MEP) have been found to impair endothelium-dependent relaxation in the rat aorta (Cheng and Kang, 1999) with short-term treatment (10 min), enhance vasoconstriction in rat smooth muscle cells with long-term treatment (18 h; Tzeng et al., 2003), induce apoptosis in macrophages (Lee and Kang, 2002), and affect metabolic enzyme activities in rat tissues (Ueng et al., 1998). Also, particles collected from scooter or motorcycle exhaust were shown to cause a certain degree of DNA damage (Kuo et al., 1998; Zhou and Ye, 1997), similar to that reported with DEP (Casellas et al., 1995; Li et al., 1996).

Although both DEP and MEP come from vehicles’ combustion, there might be some difference between the chemical components in the exhausts due to the different engine structures and the fuel used. For example, many motorcycles use two-stroke engines, which require the mixing of lubricant with the fuel. It has been shown that the addition of lubricant will lead to the incomplete combustion and mass exhaust of submicrometer carbonaceous particles (Lewtas, 1983). Chemical analysis studies demonstrated that MEP contained higher levels of benzene, xylene, and toluene than did gasoline and diesel engine exhausts (Chan et al., 1993; Jemma et al., 1995). Although both DEP and
MEP contain PAHs, their compositions are different; the major PAH components found in DEP are phenanthrenes (Barkknecht et al., 1982). Therefore, it is possible that DEP and MEP might exert different biological effects.

The aim of this study was to contribute to a better understanding of the genotoxic effect of MEP and the possible involvement of reactive oxygen species (ROS) in the induction of genotoxicity using the short-term in vitro mutagenicity bioassay (Ames Salmonella assay), the chromosome aberration test with CHO mammalian cells, and the in vivo rodent micronucleus test. We found that MEP contains chemicals that can induce genotoxicity both in vitro and in vivo and that ROS are involved in this pathway.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS), penicillin/streptomycin, Dulbecco’s modified Eagle medium (DMEM), F-12K medium, and trypsin were obtained from Gibco BRL (Grand Island, NY). Ascorbate, D (+)-glucose, and sodium chloride were obtained from Merck (Darmstadt, Germany). Acridine orange, N-acetyl-L-cysteine (NAC), catalase, glucose-6-phosphate (G6P), β-nicotinamide adenine dinucleotide phosphate (β-NADP), α-tocopherol, colcemid, 3-methylcholanthrene, and the positive control chemicals for the Ames test, 9-aminacridine, 4-nitroquinoline-N-oxide (4-NQO), 2-aminoanthracene (2AA), streptozotocin (STZ), benz(a)pyrene (BaP), and mitomycin c, were obtained from Sigma Chemical Co. (St. Louis, MO).

For the micronucleus assay in mice, MEP agents were dissolved in corn oil and mitomycin c was dissolved in PBS to give solutions with different concentrations of the tested compound for intraperitoneal (ip) injections at 10 mg/kg body weight of mice. For the in vitro Ames/Salmonella test, the compounds were dissolved in DMSO.

Collection and preparation of MEP. MEP were collected on a 0.5-mL quartz fiber filter from a 50-cm³ Yamaha two-stroke engine using 95% octane gasoline. The sampling apparatus consisted of, in sequence, a 40- (long) × 2.2-cm (diameter) stainless still dilution tube, a filter holder, and a vacuum pump. The engine was running at 150 rpm on an empty load, and the pump was set at a flow rate of 201 mL/min to collect particles for 1 h, four times daily. The filter with particulate matter was left to dry and repeatedly extracted with methanol, four times under sonication. The washed particles and organic components absorbed on the filter were pooled, and the methanol was removed in a vacuum evaporator. The final residues, the MEP and methanol extract, were collected and kept desiccated at −20°C. Approximately 32.7 μg of final residue (MEP) could be derived from 11 of motorcycle exhaust. In our extraction, methanol was used to wash and bring down the particles from the filter. After removing the methanol, the final MEP residue should contain the chemicals as well as the particles absorbed on the filter. MEP were then dissolved in DMSO or corn oil before being added to the culture medium or intratracheally instilled solution.

Experimental animals. For the Ames test, Wistar rats (200 g) were used for preparation of the liver microsomal (S9) fraction. For the micronucleus test, male ICR mice, aged 8 to 9 weeks and weighing 30–40 g, were employed. All animals were purchased from the Animal Center of the College of Medicine, National Taiwan University. The animals were allowed at least a 1-week acclimation period in their animal quarters with air conditioning and a 12-h light/dark cycle. All animal treatments were approved by the Institution Animal Care and Use Committee (IACUC) of the College of Medicine, National Taiwan University, which follows the Animal Welfare Protection Act of the Department of Agriculture, Executive Yuan, Taiwan.

S9 fraction preparation. Rat liver S9 used for metabolic activation was prepared as described previously (Maron and Ames, 1983; Matsuoka et al., 1979). To obtain the liver microsomal fraction, each of the Wistar rats was injected ip with 3-methylcholanthrene (30 mg/kg body weight) every day, and 4 days later the rats were killed by cervical dislocation. The livers were homogenated, diluted 1:4 with 0.15 M KCl, and centrifuged for 10 min at 9000 × g. The supernatant was pulled and diluted (giving a protein concentration of 30 mg/ml), frozen in small aliquots, and stored at −70 to −80°C until use. The final preparation of the metabolizing system (S9 mixture) was made in accordance with the protocol of Ames et al. (1975). The composition and final concentrations of the S9 mix used for the CHO-K1 cell chromosome aberration test were as follows: glucose-6-phosphate, 4.4 mM; NADP, 0.84 mM; KCl, 30 mM; NaHCO3, 0.032%; and S9 fraction, 10%.

Ames Salmonella/microsome test. The method we used followed the recommendations of Maron and Ames (1983) and Organization for Economic Cooperation and Development (OECD) guidelines (1997). The Salmonella typhimurium bacteria and histidine auxotrophic strains TA98, TA100, and TA102 were obtained from MOLTOX (Molecular Toxicology, Annapolis, MD) and grown for 14 h at 35 ± 2°C with continuous shaking. Bacteria were grown to a density of 1 to 2 × 10⁸ cells/ml with OD600 absorbance of 0.2–0.3. Top agar, containing 2 mL of heated agar, 0.1 mL of test chemical, 0.1 mL of bacteria, and 0.5 mL of S9 solution, was mixed up and added to three different minimal glucose agar plates. All plates were incubated at 37°C for 48 h, and the number of bacteria colonies was determined. The entire experiment was replicated again on a different day with a total of six plates for each concentration of MEP with and without S9. S9 liver cell extracts contain enzymes that may activate the potential mutagen. Each tester strain was routinely checked to confirm its features for optimal response to known mutagenic chemicals as follows: 4-NQO (0.5 μg/plate), mitomycin c (0.5 μg/plate), and 2AA (5 μg/plate). BaP (10 μM) and STZ (0.5 mM) were used as ROS-dependent positive controls with and without S9, respectively. A test compound was judged to be mutagenic in the plate test if it produced, in at least one concentration and one strain, a response equal to twice (or more) of the control incidence with a dose-response relationship considered to be positive (De Serres and Shelby, 1979; Suter et al., 2002). The only exception was strain TA102, which has a relatively high spontaneous revertant number, where an increase by a factor of 1.5 above the control level was taken as an indication of a mutagenic effect.

Chromosomal aberrations. Chinese hamster ovary epithelial cells (CHO-K1, ATCC; CCL-61) were plated into 6-cm dishes at 5 × 10⁵ cells/plate for the 24-h treatment group. After overnight incubation, cells were treated with DMSO (solvent), mitomycin c (1 μg/ml), BaP pyrene (5 μg/ml), and various concentrations of S9 for 3 h with or without S9. Then, 3 h after the end of the treatment time, 0.1 μg/ml colcemid was administered, and metaphase chromosomes were prepared as described (Tsutsi et al., 1983). After trypsinization, cells were treated with 0.9% sodium citrate at 37°C for 10 min, fixed in Carnoy’s solution (methanol:acetic acid, 3:1), and spread on glass slides by the air-drying method. Specimens were stained with a 3% Giemsa solution in 0.07 M phosphate buffer (pH 6.8) for 30 min. For determination of both chromosome aberrations, 100 metaphases per experimental group were scored. Structural chromosome aberrations observed in each experimental group were classified into seven types as follows: chromosome-type gap (G), chromosome-type break (B), chromosome-type ring (R), chromosome-type dicentric (D), chromatid-type break (d), chromatid-type exchange (e). Achromatic lesions greater than the width of the chromatid were scored as gaps unless there was displacement of the broken piece of chromatid. If there was displacement, it was recorded as a break.

Micronucleus assay. The micronucleus assay from peripheral blood cells was performed as previously described (Hayashi et al., 1990). The number of micronucleated cells was counted in 1000 reticulocytes (RETS) per animal. Slides were analyzed using a fluorescent microscope with a combination of a blue excitation (e.g., 488 nm) and a yellow-to-orange barrier filter (e.g., 515 nm long pass), with a ×100 objective for RETs.

Analysis of ROS production by flow cytometry. Intracellular ROS generation was measured by a flow cytometer with an oxidation-sensitive DCFH-DA fluoroprobe (Rothe and Valet, 1990). DCFH-DA (2′,7′-dichlorofluorescin diacetate) is a nonfluorescent compound that is freely taken up into cells and...
hydrolyzed by esterase by removing the DA group. This nonfluorescent molecule (DCFH) is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. When DCFH-DA was deacetylated and oxidized to form the fluorescent compound, fluorescence intensity was increased in a concentration-dependent manner. We stained $2 \times 10^6$ CHO-K1 cells with 20 $\mu$g/ml DCFH-DA for 30 min at 37°C in the dark. Cells were then collected after PBS washing for fluorescence measurement. The level of intracellular ROS was determined with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. For each treatment, 10,000 cells were counted and the experiment was performed in triplicate.

Statistical analysis. Data were expressed as the mean ± SEM for the number of experiments indicated. Statistical analysis of the data was performed by Student’s $t$-test, and $p < 0.05$ was considered significantly different. The values of IC$_{50}$ were calculated and obtained from five regression lines; each regression line was constructed of at least five points. The values of inhibition of these points ranged from 20 to 80%.

RESULTS

Mutagenicity of MEP

The mutagenicity of MEP was examined by the Ames method (Maron and Ames, 1983). The assay was carried out in vitro using three histidine-requiring strains of Salmonella typhimurium (TA98, TA100, and TA102) with and without a metabolic-activating enzyme (S9). Each strain of Salmonella was treated with MEP at 1, 10, 100, 1000, and 2000 $\mu$g/plate, respectively. Our data showed that MEP did not increase colony formation in strains TA98, TA100, and TA102 without the S9 mix (Table 1) at concentrations of up to 2 mg/plate. On the other hand, in the presence of S9, the number of revertants in MEP-treated plates was concentration-dependently increased in TA98, TA100, and TA102 strains (Table 1). Pretreatment with antioxidants, including $\alpha$-tocopherol (3.5 $\mu$M/plate), ascorbate (100 $\mu$M/plate), NAC (1 mM/plate), and catalase (1000 U/plate), showed different potencies, but all attenuated the colony formation induced by MEP (1000 $\mu$g/plate) in TA98, TA100, and TA102 strains with the S9 mix (Table 2). In particular, $\alpha$-tocopherol significantly exerted attenuation of MEP-induced colony formation, even up to 30–40% inhibition of these testing strains. Two well-known, ROS-dependent mutagens, STZ and BaP, were used as positive controls (Table 1). The effects of antioxidants on these positive compounds have been widely reported. STZ can induce ROS-dependent mutagenesis without metabolic activation (Bedoya et al., 1996; Nakatsuka et al., 1988; Takasu et al., 1991); BaP is a promutagen (Kim and Wells, 1996; Wells et al., 1997; Winn and Wells, 1997).

### TABLE 1
Revertants in Three Strains of *Salmonella typhimurium* Treated with Different Concentrations of MEP in the Absence and Presence of a Metabolic-Activating Enzyme (S9)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Negative control</th>
<th>Positive control</th>
<th>STZ (0.5 mM)</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA98</td>
<td>20 ± 2</td>
<td>251 ± 34</td>
<td>98 ± 10</td>
<td>20 ± 1</td>
<td>21 ± 2</td>
<td>24 ± 2</td>
<td>31 ± 6</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>TA100</td>
<td>79 ± 1</td>
<td>852 ± 59</td>
<td>3989 ± 252</td>
<td>76 ± 5</td>
<td>86 ± 0</td>
<td>101 ± 8</td>
<td>79 ± 7</td>
<td>81 ± 17</td>
</tr>
<tr>
<td>TA102</td>
<td>150 ± 7</td>
<td>1264 ± 96</td>
<td>805 ± 60</td>
<td>167 ± 5</td>
<td>179 ± 16</td>
<td>231 ± 6</td>
<td>175 ± 1</td>
<td>172 ± 5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Negative control</th>
<th>Positive control</th>
<th>BaP (10 $\mu$M)</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA98</td>
<td>28 ± 2</td>
<td>3802 ± 129</td>
<td>684 ± 50</td>
<td>37 ± 1</td>
<td>54 ± 13</td>
<td>250 ± 53</td>
<td>387 ± 42</td>
<td>407 ± 73</td>
</tr>
<tr>
<td>TA100</td>
<td>92 ± 6</td>
<td>3473 ± 385</td>
<td>976 ± 42</td>
<td>92 ± 1</td>
<td>107 ± 13</td>
<td>287 ± 13</td>
<td>291 ± 52</td>
<td>459 ± 103</td>
</tr>
<tr>
<td>TA102</td>
<td>250 ± 12</td>
<td>1699 ± 207</td>
<td>857 ± 61</td>
<td>248 ± 14</td>
<td>283 ± 22</td>
<td>310 ± 22</td>
<td>478 ± 89</td>
<td>637 ± 68</td>
</tr>
</tbody>
</table>

Note. Values are presented as the mean ± SE ($n \geq 6$). The negative control consisted of 2 $\mu$l DMSO/plate. The positive control in the –S9 plate consisted of TA98, 2 $\mu$g/plate 4-nitro-o-phenylenediamine; TA100 and TA102, 0.5 $\mu$g/plate mitomycin C. The positive control in the +S9 plate consisted of 5 $\mu$g/plate 2-aminoanthrene. The ROS-dependent positive controls were streptozotocin (STZ, 0.5 mM without S9) and benzo(a)pyrene (BaP, 10 $\mu$M with S9).

$p < 0.05$ versus negative control.

$p < 0.01$ versus DMSO.

$p < 0.05$ versus DMSO.
Induction of Chromosome Aberrations by MEP in CHO Cells

The in vitro effect of MEP on chromosomes was studied with CHO-K1 cells, and the results of CHO-K1 cell chromosome analysis are given in Table 3. The incidence of CHO-K1 cells with structural chromosomal aberrations significantly increased in cells treated with BaP (5 μg/ml; 13.1 ± 2.2%, p < 0.001; Table 3) and mitomycin c (1 μg/ml; 9.8 ± 2.2%, p < 0.001; Table 3) with and without S9, respectively. Both in the absence and presence of S9, MEP (0.5, 5, and 50 μg/ml) concentration-dependently increased structural chromosomal aberrations at 3 h of treatment compared to the solvent control (0.1% DMSO, Table 3). We also found that MEP- (5 and 50 μg/ml) induced chromosomal aberration was potentiated up to 2-fold by the addition of S9 (Table 3). Pretreatment with the antioxidants α-tocopherol (3.5 μM), ascorbate (100 μM), catalase (1000 U/ml), and NAC (1 mM) yielded different potencies, but all significantly attenuated the increase in aberrant cells induced by MEP (50 μg/ml) in the S9-treated group, though not in the group without S9 (Table 4).

In Vivo Induction of Micronuclei by MEP

Results of the micronucleus assay of ICR mice treated with different doses of MEP and the positive control are shown in Table 5. Mice injected (ip) with mitomycin C (1 mg/kg body weight) showed a significant increase in the frequency of RETs at 24 (12.2 ± 1.4) and 48 h (36.5 ± 1.6) of treatment compared to control (1.2 ± 0.2 and 1.4 ± 0.5, respectively). Intratracheal instillation with various doses of MEP (160, 200, and 240 mg/kg body weight) in mice significantly increased the formation of RETs at both 24 and 48 h, with a return to basal level at 72 h after treatment (Table 5). Cotreatment with antioxidants α-tocopherol (75 mg/kg body weight) and ascorbate (1 g/kg body weight) inhibited the MEP- (200 mg/kg body weight) induced increase of RETs formation only at 48 h (Table 5).

MEP Induced ROS Formation in CHO-K1 Cells

ROS generation induced by MEP was examined using a DCFH-DA fluorescence probe. The fluorescence intensity of oxidative DCF in reaction with various concentrations of MEP was determined in CHO-K1 cells. We found that MEP increased the fluorescence intensity as ROS level in a concentration-dependent manner (Fig. 1). MEP- (50 μg/ml) induced fluorescence intensity was potentiated up to 2-fold compared to control.

DISCUSSION

Large numbers of people in the world continue to be exposed to pollutant mixtures containing known or suspected carcinogens. Epidemiologic studies over the last 50 years suggest rather consistently that general ambient air pollution, mainly due to the incomplete combustion of fossil fuels, may be responsible for increased rates of lung cancer (Cohen and Pope, 1995). These substances are present as components of complex mixtures, which may include carbon-based particles that absorb organic compounds, oxidants such as ozone, and sulfuric acid in aerosol form (Cass et al., 1984; Dockery et al., 1993). In Taiwan, the combustion of fossil fuels for power generation and transportation (especially motorcycles), also the primary source of many organic and inorganic compounds, oxidants, and acids, contributes heavily to particulate air pollution. In this study, we found MEP extract was mutagenic in both in vitro and in vivo experimental assays.
Over the years, the Ames test has been used worldwide as an initial screening tool to determine the mutagenic potential of new chemicals and drugs, because there is a high predictive value for rodent carcinogenicity when a mutagenic response is obtained (McCann et al., 1975; Sugimura et al., 1976; Zeiger et al., 1990). Our data showed that MEP contains chemicals that are mutagenic to Salmonella strains TA98, TA100, and TA102 (Myriam et al., 1990). The involvement of ROS. Strain TA102 was developed containing AT base pairs at the hisG428 mutant site, and the mutation is reverted by mutagens that cause oxidative damage (Myriam et al., 2000; Niittykoski et al., 1995). The involvement of ROS in MEP-induced mutation was further evidenced by the alleviation of mutation with the use of antioxidants including α-tocopherol, ascorbate catalase, and NAC. Alpha-tocopherol and ascorbate have similar effects in preventing MEP-induced mutation with the use of antioxidants including α-tocopherol, ascorbate catalase, and NAC. The organic component of DEP has also been shown to generate ROS that produce 8-hydroxydeoxyguanosine (8-OHdG) in cell culture (Tsurudome et al., 1999). Our data showed the mutagenic effect on TA102, providing proof of the involvement of ROS. Strain TA102 was developed containing AT base pairs at the hisG428 mutant site, and the mutation is reverted by mutagens that cause oxidative damage (Myriam et al., 2000; Niittykoski et al., 1995). The involvement of ROS in MEP-induced mutation was further evidenced by the alleviation of mutation with the use of antioxidants including α-tocopherol, ascorbate catalase, and NAC. Alpha-tocopherol and ascorbate have similar effects in preventing MEP-induced mutation with the use of antioxidants including α-tocopherol, ascorbate catalase, and NAC.

### Table 3

Chromosome Aberrations of CHO-K1 Cells Treated with MEP

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Aberrant cells (%)</th>
<th>G</th>
<th>B</th>
<th>D</th>
<th>R</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent control (DMSO)</td>
<td>3.1 ± 0.1</td>
<td>1.6 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>MMC (1 µg/ml)</td>
<td>9.8 ± 2.2</td>
<td>0</td>
<td>5.3 ± 0.3</td>
<td>0.3 ± 0.3</td>
<td>0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>MEP (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>6.0 ± 2.2</td>
<td>2.7 ± 1.5</td>
<td>0.5 ± 0.3</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.8 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>6.2 ± 1.9</td>
<td>1.3 ± 0.9</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0</td>
<td>3.7 ± 1.9</td>
</tr>
<tr>
<td>50</td>
<td>10.0 ± 2.0</td>
<td>1.3 ± 1.3</td>
<td>0.7 ± 0.3</td>
<td>1.0 ± 0.5</td>
<td>2.0 ± 1.0</td>
<td>2.3 ± 2.3</td>
</tr>
<tr>
<td>With S9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solvent control (DMSO)</td>
<td>3.4 ± 1.0</td>
<td>1.6 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>0.1 ± 0.1</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td>BaP (5 µg/ml)</td>
<td>13.1 ± 2.2</td>
<td>3.8 ± 1.3</td>
<td>0.5 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 0.4</td>
<td>4.0 ± 1.8</td>
</tr>
<tr>
<td>MEP (µg/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>7.0 ± 2.9</td>
<td>1.5 ± 0.7</td>
<td>1.2 ± 1.2</td>
<td>0.2 ± 0.2</td>
<td>0.5 ± 0.5</td>
<td>3.0 ± 1.5</td>
</tr>
<tr>
<td>5</td>
<td>13.3 ± 2.0</td>
<td>3.2 ± 1.6</td>
<td>1.3 ± 1.3</td>
<td>1.7 ± 1.4</td>
<td>0.8 ± 0.8</td>
<td>3.8 ± 2.0</td>
</tr>
<tr>
<td>50</td>
<td>19.8 ± 3.5</td>
<td>3.8 ± 2.0</td>
<td>3.0 ± 2.5</td>
<td>2.0 ± 2.0</td>
<td>2.0 ± 1.0</td>
<td>5.7 ± 3.0</td>
</tr>
</tbody>
</table>

**Note.** Values are presented as the mean ± SE (n = 3). MEP was dissolved in DMSO, and the solvent control did not exceed 0.1%. Mitomycin C (MMC) was the positive control in the medium without S9, and BaP was the positive control in the medium with S9. G, chromosome gap; B, chromosome break; D, dicentric; R, ring; g, chromatid gap; b, chromatid break; e, exchange.

*p < 0.001 versus DMSO.

*p < 0.01.

*p < 0.05.
TABLE 4
Chromosome Aberrations of MEP-Treated CHO-K1 Cells in the Presence of Antioxidants

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Aberrant cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h without S9</td>
</tr>
<tr>
<td>Solvent control (DMSO)</td>
<td>3.3 ± 0.3</td>
</tr>
<tr>
<td>Positive control</td>
<td>10.7 ± 0.9**</td>
</tr>
<tr>
<td>MEP (50 μg/ml)</td>
<td>10.0 ± 2.0**</td>
</tr>
<tr>
<td>+ α-tocopherol (3.5 μM)</td>
<td>7.0 ± 1**</td>
</tr>
<tr>
<td>+ ascorbate (100 μM)</td>
<td>9.0 ± 0</td>
</tr>
<tr>
<td>+ catalase (1000 U/ml)</td>
<td>8.0 ± 1**</td>
</tr>
<tr>
<td>+ NAC (1 mM)</td>
<td>9.5 ± 0.5**</td>
</tr>
</tbody>
</table>

Note. Values were presented as the mean ± SE (n = 3). MEP was dissolved in DMSO, and the solvent control did not exceed 0.1%. MMC was the positive control in the medium without S9, and BaP was the positive control in the medium with S9. G, chromosome gap; b, chromosome break; D, dicentric; R, ring; g, chromatid gap; b, chromatid break; e, exchange.

TABLE 5
Micronucleus Formation in Peripheral Blood Cells of Mice Treated with MEP and the Effects of Antioxidants In Vivo

<table>
<thead>
<tr>
<th></th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>MMC (1 mg/kg)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>MEP (160 mg/kg)</td>
<td>12.2 ± 1.4a</td>
</tr>
<tr>
<td>MEP (200 mg/kg)</td>
<td>2.8 ± 0.6b</td>
</tr>
<tr>
<td>MEP (240 mg/kg)</td>
<td>5.0 ± 0.3a</td>
</tr>
</tbody>
</table>

|                          |          |          |          |
| Control                  |          |          |          |
| MEP (200 mg/kg)          | 1.2 ± 0.2| 1.4 ± 0.5| 1.0 ± 0.2|
| MEP (200 mg/kg) + ascorbate (1 g/kg) | 2.7 ± 0.5d| 5.6 ± 0.4d| 2.1 ± 0.5 |
| MEP (200 mg/kg) + α-tocopherol (75 mg/kg) | 2.0 ± 0.8 | 2.0 ± 0.2c | 1.5 ± 0.5 |

Note. Values were presented as the mean ± SE (n = 3). The control consisted of 1% Tween 80 in DMSO:PBS (1:9). Ascorbate (1 g/kg in water) and α-tocopherol (75 mg/kg in corn oil) were used for pretreatment by an ip injection for 30 min.

The addition of S9 greatly enhanced the effect of the MEP-induced aberrations (19.8 ± 3.5, p < 0.001 at 50 μg/ml; Table 3). These data suggested that MEP contains chemicals that can induce chromosome aberrations both with and without metabolic activation in CHO cells. Previously, Kuo et al. (1998) also showed that MEP induced genotoxicity in a ROS-dependent manner by using cell-junction communication as an experimental model. Pretreatment with antioxidants selectively attenuated MEP-induced aberration of cells in the S9-treated group (Table 4). This result indicated that MEP might contain chemicals that exert a mutagenic effect independent of ROS and bioactivation in CHO cells. The mechanistic detail still requires further investigation.

The clastogenic effects of MEP were also detected with the in vivo micronucleus test in mice peripheral red blood cells. Micronuclei scoring is based on the observation that displaced chromatin, resulting from chromosome loss or breakage, may fail to be incorporated into daughter nuclei as a cell divides. The resulting micronucleus is found in the cytoplasm. During erythropoiesis, an erythroblast expels its main nucleus to become a reticulocyte (RET), while the micronuclei remain in the cytoplasm. The newly formed RET is then released from the bone marrow into the circulating bloodstream, where it develops into a normochromatic erythrocyte (NCE). Elevations in the frequency of micronuclei are indicative of genotoxic activity (Hayashi et al., 2000). MEP dose-dependently increased the micronucleus formation at 24 and 48 h after treatment, and this effect was slightly reversed to basal level at 72 h. Obviously, the in vivo study showed that the effect is reversible or can be repaired after 72 h. Intraperitoneal injection with ascorbate (1 g/kg in water) and α-tocopherol (75 mg/kg in corn oil), 30 min before MEP (200 mg/kg) treatment, greatly reduced the micronucleus formation at 48 h in the treated group.

Micronuclei can arise from acetic fragments induced by a substance causing chromosomal breakage (clastogens) as well as aneuploidy (aneugens). However, with the conventional micronucleus test, it is impossible to distinguish between these two events. We cannot rule out the possibility that MEP might be an aneugen in this study. The reason the antioxidants showed the most obvious protecting effect at 48 h might be because the micronucleus showed the most dramatic change at 48 h. It is also possible that the antioxidants only have a partial effect, as seen with the Ames and chromosome tests. The basal micronucleus observed at all time points, which was not inhibited by the antioxidants, might be caused by some chemicals in MEP that damage the chromosome independently of ROS formation.

There are some possible sources that can account for the origination of ROS generation. They include the following:

1. Among the > 100 chemicals that are present in MEP (Chan et al., 1993; Zhou and Ye, 1997), such as PAH, nitroderivatives of PAH, and oxygenated PAH derivatives (ketones, quinines, and dienes) are candidate chemicals that may contribute to ROS generation (Alsborg et al., 1985; Anderson et al., 1998; Li et al., 1996; Schuetzle et al., 1981).
2. Quinone is reduced to semiquinone radicals by microsomally localized cytochrome P450 reductases (Chesis et al., 1993; Zhou and Ye, 1997), such as PAH, nitroderivatives of PAH, and oxygenated PAH derivatives (ketones, quinines, and dienes) are candidate chemicals that may contribute to ROS generation (Alsborg et al., 1985; Anderson et al., 1998; Li et al., 1996; Schuetzle et al., 1981).
These semiquinones group together, thereby initiating a futile redox cycle (Chesis et al., 1984; Monks et al., 1992).

3. PAH may contribute to further ROS production during cytochrome P4501A1-dependent transformation.

4. In addition to the contribution of organic chemical compounds, that of metal ions, such as Fe^{2+}, may relate to ROS generation.

5. Mitochondria have been implicated in the induction of apoptosis by a growing list of pro-oxidative chemicals, including redox-cycling quinines and PAH (Segura-Aguilar et al., 1998; Yamaguchi et al., 1996; Zoratti and Szabo, 1995).

ROS generation has been linked to the mutagenic effects of DEP chemicals (Ichinose et al., 1997). Although these MEP-associated chemicals need to be identified, we know that MEP contain PAHs, which have potentially harmful effects on humans. The PAH components can react with nitrous oxides in the air, such as NO2 and NO, and form nitro-PAH. Recent studies demonstrated that nitro- and dinitropyrene play a minor role in air particulate mutagenicity (Crebelli et al., 1991).

Our study provides evidence of the potentially genotoxic effects of MEP extract both in vitro and in vivo. Although bioactivation and ROS generation were shown to play major roles in the genotoxic effects that we observed, MEP also contains directly acting chemicals and chemicals that act independently of ROS. The impact of this toxic effect of MEP on the health of people in Taiwan is worthy of emphasis and further investigation.

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REFERENCES


