



Comparative *In Vitro* Biological Toxicity of Four Kinds of Air Pollution Particles

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Accumulating epidemiological evidence indicates that exposure to fine air pollution particles (APPs) is associated with a variety of adverse health effects. However, the exact physicochemical properties and biological toxicities of fine APPs are still not well characterized. We collected four types of fine particle (FP) (diesel exhaust particles [DEPs], natural organic combustion [NOC] ash, synthetic organic combustion [SOC] ash, and yellow sand dust [YSD]) and investigated their physicochemical properties and *in vitro* biological toxicity. DEPs were almost entirely composed of ultrafine particles (UFPs), while the NOC, SOC, and YSD particles were a mixture of UFPs and FPs. The main elements in the DEPs, NOC ash, SOC ash, and YSD were black carbon, silicon, black carbon, and silicon, respectively. DEPs exhibited dose-dependent mutagenicity even at a low dose in *Salmonella typhimurium* TA 98 and 100 strains in an Ames test for genotoxicity. However, NOC, SOC, and YSD particles did not show any mutagenicity at high doses. The neutral red uptake assay to test cell viability revealed that DEPs showed dose-dependent potent cytotoxicity even at a low concentration. The toxicity of DEPs was relatively higher than that of NOC, SOC, and YSD particles. Therefore, these results indicate that among the four FPs, DEPs showed the highest *in vitro* biological toxicity. Additional comprehensive research studies such as chemical analysis and *in vivo* acute and chronic inhalation toxicity tests are necessary to determine and clarify the effects of this air contaminant on human health.

Key words: Air pollution particle, Physicochemical properties, Mutagenicity, Cytotoxicity

INTRODUCTION

The Organisation for Economic Co-operation and Development (OECD) indicated in their report, "The Economic Consequences of Outdoor Air Pollution", published on June 09, 2016, that there were approximately 3 million premature deaths related to outdoor air pollution worldwide in 2010 and projected that the incidence will be 6–9 million in 2060. Particularly, in Korea, the number of deaths caused by outdoor air pollution per million was estimated to be 259

in 2010, but it is currently predicted to be 1,109 and furthermore, the incidence is increasing towards a potentially alarming world record for the nation.

Ultrafine particles (UFPs) of air pollutants, which are less than 2.5 μm in diameter, effectively bypass the deposition process of the nasal and trachea bronchial mucus. This can result in a higher proportion of particles reaching the lower respiratory tract, which greatly affect the human body, including respiratory function (1). Although fine particle (FP)-induced injuries and damages appear to occur to varying degrees, particle-mediated inflammation may likely be the predominant mechanism of respiratory tract injury and cardiovascular effects (2).

Gas phase and particulate matter emitted following incomplete combustion and burning of organic or inorganic matter worsen the air quality, have a deleterious effect on the respiratory system, and are commonly known to cause various respiratory diseases such as bronchitis, asthma, chronic obstructive pulmonary disorder (COPD), and lung cancer

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(3). Particulate matters composed of a diverse mixture of FPs and UFPs, containing metals, soot, tar, black carbon, and carbonaceous materials, have a relatively large surface area to absorb toxic materials (4). Following absorption, these materials can be subsequently delivered to the bronchus or germinal cells where they adhere to the lungs and induce mutagenicity and carcinogenesis (5).

Combustion-derived diesel engine exhaust from cars, trucks, buses, and recreational vehicles contains black carbon and hydrocarbons (C₁₄-C₃₅), as well as heterocyclic polycyclic aromatic hydrocarbons (PAHs) and the derivatives of these substances. In addition, it contains acids, alcohols, esters, anhydrides, ketones, nitriles, quinones, sulfonates, organic halogens, inorganic sulfates and nitrates, and heavy metals (6), which are known to cause respiratory diseases such as inflammation, lung cancer, asthma, and allergies (7).

Natural organic combustion (NOC) smoke is emitted from forest fires and materials burnt to heat rooms and for cooking, including biomass fuels such as woods, pellets, and grain husks, which are time-honored traditional practices. The particle emissions from NOC are nano-sized and contain oxidation products such as metals, black carbon, PAHs, and heterocyclic compounds (8,9). Synthetic organic combustion (SOC) smoke emitted from industrial waste incinerators also contain fly ashes, metals, halogenated PAHs, dioxins, and heterocyclic compounds (10). Yellow sand dust (YSD) contains particles that are mostly less than 5 µm in diameter and consists of heavy metals, acidic substances, inorganic sulfates and nitrates, bacteria, and fungi (11).

Furthermore, the physical characteristics and chemical analysis of a large number of toxic components of air pollutants with potentially harmful health effects have been widely reported (12,13). However, comparative *in vivo* and *in vitro* toxicological evaluations of these substances have rarely been conducted. Therefore, in this study, we collected FP samples of the air pollutants, diesel exhaust particles (DEPs), NOC ashes, SOC ashes, and YSD, to investigate their physical characteristics and perform chemical elemental analysis. Furthermore, we compared their *in vitro* toxicological activity, such as mutagenicity and cell toxicity, to evaluate their relative contribution to the associated human health effects.

MATERIALS AND METHODS

DEP collection. DEPs were provided by the National Institute for Environmental Studies in Tsukuba Science City, Japan. The DEP samples were collected using a glass filter in a constant-volume sampler system attached to the end of a dilution tunnel that was connected to a four-cylinder diesel engine (Isuzu Automobile Company, Tokyo, Japan), which operated at 1,500 rpm under a 10 torque (kg·m) load, as described previously (14). The particles were stored at

-70°C in a deep freezer until they were used for the experiments.

NOC ash collection. Dried rice husks were purchased at an animal feed store to prepare typical NOC ashes. Briefly, 1,115.33 g of dried rice husk was burned to ashes on the clean bottom of a clay fire pot, and the ashes were collected, strained using a standard testing sieve (250 µm), and then stored at -0°C in a deep freezer until used.

SOC ash collection. Synthetic organic matter (152.03 g) consisting of 34.68 g black plastic bags, 34.68 g polyethylene terephthalate bottles and pastry wrappers, and Styrofoam were purchased at area markets and burned to ashes on the clean bottom of a clay fire pot. The ashes were collected, strained using a standard testing sieve (250 µm), and stored at -70°C in a deep freezer until used.

YSD collection. The YSD was collected from the rooftop of the S 21-1 building of Chungbuk National University. White plastic mats were laid on the rooftop in the early morning of the day on which a YSD storm warning was forecasted by the government. The YSD that sank naturally under the influence of gravity was collected and stored at -70°C in a deep freezer until they were used for the experiments.

Physicochemical properties. The morphology and chemical composition of four FP types were determined using a high-resolution scanning electron microscopy (HR-SEM). The equipment used was the Quanta 200 FEG scanning electron microscope (FEI, Quanta FP2017/11), equipped with a chemical composition analysis system based on energy dispersive X-ray analysis (EDAX). The collected samples were mounted on an aluminum stud using carbon tape. The samples were then coated with gold using a sputter coating unit (MSD-1S, VD Co., Japan), with the vacuum set at 10 Pa for 10 sec. The acceleration potential used was 30 kV, and the images were captured at the desired magnification.

Bacteria and culture medium. The *Salmonella typhimurium* TA 98 and TA 100 tester strains used for evaluating frame-shift and base-pair substitution type mutations, respectively, were provided by the Korea Institute of Toxicology (KIT). The strains were grown with 2.5% nutrient liquid in a spinner flask at 37°C in a shaking incubator maintained at a speed of 80 rpm for 10 hr. The minimal glucose agar plate was prepared by combining 1.5% Bacto agar, Vogel-Bonner medium E, and 2% glucose, while the top agar was prepared by the addition of 0.6% agar, 0.5% sodium chloride (NaCl), and 0.05 mM histidine-biotin. In addition, we analyzed the histidine requirement and conducted *uvrB* mutation maintenance, R-factor maintenance, *rfa* mutation, and spontaneous mutation tests. Both strains

were used for these tests.

Mutagenicity assay. Mutagenicity was evaluated using the TA 98 and TA 100 *S. typhimurium* strains according to the method described by Maron and Ames (15). The test materials were incorporated in the plates, similar to the plate-incorporation method. The positive control groups contained 0.5 µg/plate of direct mutagen 4-NQO, 1 µg/plate of sodium azide (SA), and 0.5 µg/plate of indirect mutagen 2-AA. Dimethyl sulfoxide (DMSO) was also added to the agar plate as a negative control substance. In the test, we used 500 µL of 0.2 M sodium phosphate buffer (pH 7.4) instead of the S-9 mix for the group tested in the absence of hepatic metabolic activations; 500 µL S-9 mix was included in the hepatic metabolic activation group, which required the indirect mutagen. Then, 100 µL of the culture medium ($1-2 \times 10^9$ cells/mL) and the FPs were placed into a 5-mL test tube. The mixture was mixed after replacing the cap tubes, and then it was poured into the culture medium and incubated for 48 hr in an incubator at 37°C. Finally, the number of revertant colonies on the plate was counted.

Determination of concentration. To set the maximum concentration used in this test, a 10 mg/mL stock solution of total particulate matter (TPM) from each of the extracted FPs was used. Finally, we set the maximum concentration at 1 mg/plate. After the test, the reverse mutation colonies that exhibited a capacity correlation and reproducibility, as well as an increase two times that of the negative controls were considered to show positive mutations. If the background lawn was decreased compared with that of the negative controls, severe toxicity was confirmed. We established the optimal concentration based on these results.

Cytotoxicity (cell viability) test. The percentage cell viability was determined using the neutral red uptake assay to evaluate cytotoxicity according to the method of Ellen and James (16). CHO-K1 cells were seeded in 96-well plates at a density of 1.0×10^4 cell per well with 100 µL of the culture medium. The 96-well plates were placed in a humidified atmosphere at 37°C (5% CO₂ and 95% air) for 24 hr. After aspirating the culture medium, 200 µL of the diluted samples was dispensed into each well, followed by 24 hr of incubation. At the designated times, diluted samples were replaced with 200 µL of neutral red solution (50 µg/mL in the culture medium without fetal bovine serum [FBS]) for 3 hr.

The neutral red solution was aspirated, 200 µL of the wash-fix solution (1% [v/v] formalin) was added to each well, and then the wash-fix solution was aspirated within 1 min. Following this, 200 µL of neutral red extract solution was added to each well, and the 96-well plate was placed on a microplate plate shaker for at least 10 min. The optical density of each well was measured using a micro-

plate reader (Bio-Tek, USA) at an absorption wavelength of 540 nm. The dose-response curve was constructed using the test sample values expressed as a percentage of the control value (the absorbance of neutral red from UFP-treated cells was divided by corresponding control cell values). The half-maximal effective concentration (EC₅₀) value (the dose that induced 50% inhibition of cell viability) was extrapolated from the dose-response curve.

Statistical analysis. The experimental data were presented as the mean ± standard deviation (SD) of at least three replicates. A one-way analysis of variance (ANOVA) was conducted to compare the results of each UFP. The Student's *t*-test was used for the statistical analysis of the differences and $p \leq 0.05$ was considered statistically significant.

RESULTS

Physicochemical properties.

• **DEP:** The mean particle size of the DEP samples measured using an electron microscope was mostly < 4 µm (Fig. 1). Furthermore, most DEPs were < 3 µm in diameter, completely black, and spherical. These results are in accordance with the results of Sagai *et al.* (14). Elemental analysis of the DEPs showed that they contained carbon at the highest level, followed by oxygen, zirconium, silicon, palladium, sulfur, silver, and calcium. The results reported by Surawski *et al.* (17) showed a particle size distribution that had a strong dependency on the biodiesel blend percentage. In addition, the median particle diameter decreased as the biodiesel blend percentage increased. We confirmed that the DEP samples were produced by incomplete oxidation when light oil was burned in the diesel engine in our experiments.

• **NOC:** The NOC particle size had a very wide range, from 4 to 10 µm in diameter. The rice husks were easily burned in a fire pot without requiring any accelerant because they were dried sufficiently. Most of the NOC ash samples were nearly black and lineal. The following proportions of chemical elements were detected in the NOC samples, in descending order of atomic content: 66.52% silicon, 10.53% zirconium, 8.14% potassium, 3.87% chloride, 2.27% palladium, 2.22% sodium, 2.06% calcium, and 2.00% magnesium. The NOC process was likely complete since the carbon content was almost nonexistent in the ash samples. Faustini *et al.* (18) reported that NOC smoke generated by wildfire was associated with increased cardiovascular mortality in urban residents, and the particulate matter less than 10 microns in diameter (PM₁₀) on smoky days has a greater effect on cardiovascular and respiratory mortality than it did on other days. Naeher *et al.* (19) also reported that NOC in wood-burning stoves and fireplaces as well as wildland and agricultural fires emitted significant quantities of known health-damaging pollutants, including several carcinogenic compounds.

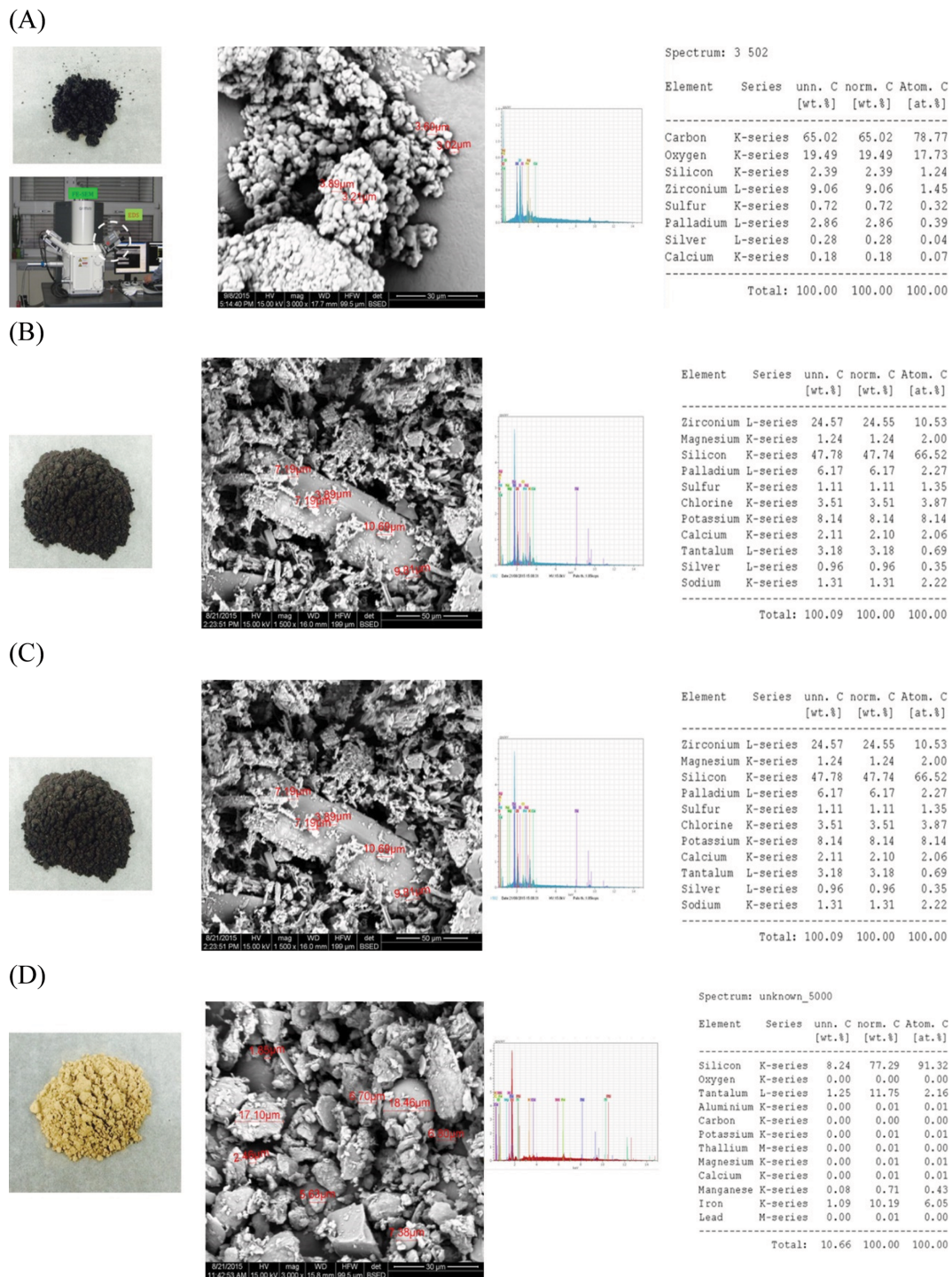


Fig. 1. Physiochemical properties determined using SEM and energy dispersive X-ray spectroscopic analysis of air pollutant particles. (A) DEP, (B) NOC ashes, (C) SOC ashes, and (D) YSD. SEM, scanning electron microscopy; DEP, diesel exhaust particles; YSD, yellow sand dust; NOC, natural organic combustion; SOC, synthetic organic combustion.

• **SOC:** The SOC particle size was $\geq 4.0 \mu\text{m}$. The synthetic organic matter used in this study did not burn by itself and produce thick, black smoke. Furthermore, it was completely withered by heat with poor light. Most of the SOC

ashes were brown black and liberal. The chemical elements detected in the SOC were black carbon, aluminum, silicon, zirconium, and palladium. Black carbon was present at the highest level in the SOC ashes, followed by zirconium, pal-

ladium, silicon, aluminum, and sulfur, in order of relative ratio. The SOC process was likely incomplete without a forced oxygen supply and was variable. Yang *et al.* (20) reported that the average PM_{2.5}/TPM ratio emitted from a municipal solid waste incinerator was 0.93, and the major chemical species detected in PM_{2.5} were organic carbon, chloride ions, ammonium ions, elemental carbon, and silicon, while the minor species were sulfate ions, potassium ions, sodium, potassium, nitrate, aluminum, calcium ions, zinc, calcium, copper, iron, lead, and magnesium. Fujimori *et al.* (21) reported that the chemical determinants of the generation of aromatic-Cl, such as polychlorinated biphenyls (PCBs) and chlorobenzenes, in fly ashes from municipal solid waste incineration were the precise ratios of copper, iron, lead, and zinc chlorides and oxides.

• **YSD:** The YSD particles also had a wide size range, from 1.86 to 18.46 μm. YSD is not a combustion product, but it is blown over to Korea from China on the westerlies. The YSD particles were yellow and spherical. Silicon was the most abundant element in YSD, followed by tantalum, iron, manganese, aluminum, potassium, magnesium, and calcium, in that order. Ma *et al.* (22) investigated the physi-

cochemical properties of YSD using a multiple analysis method and reported the major components to be aluminum silica, silicon dioxide, and calcium carbonate, with organic compounds and inorganic nitrates coated on the particle surfaces. Noh *et al.* (23) estimated the mass concentration of YSD based on the weight percentage (38~77%) of the mineral quartz. Compared to other particles, YSD was mainly composed of inorganic elements, and organic substances, such as fungi and bacteria, were also detected.

Mutagenicity. Fig. 2 shows the change in the number of revertant colonies following treatment of the *S. typhimurium* TA 98 strain with various concentrations of APPs. The characteristics of the TA 98 strain are useful for detecting a frame-shift type mutation. Comparing the relative mutagenicity of the four samples in the TA 98 strain showed that DEPs dose-dependently increased the revertant colony number at low concentrations. Contrastingly, the revertant colony number decreased at higher concentrations, specifically greater than 100 μg/plate.

Based on these results, we determined that DEPs at a high concentration were highly toxic in the TA 98 strain culture

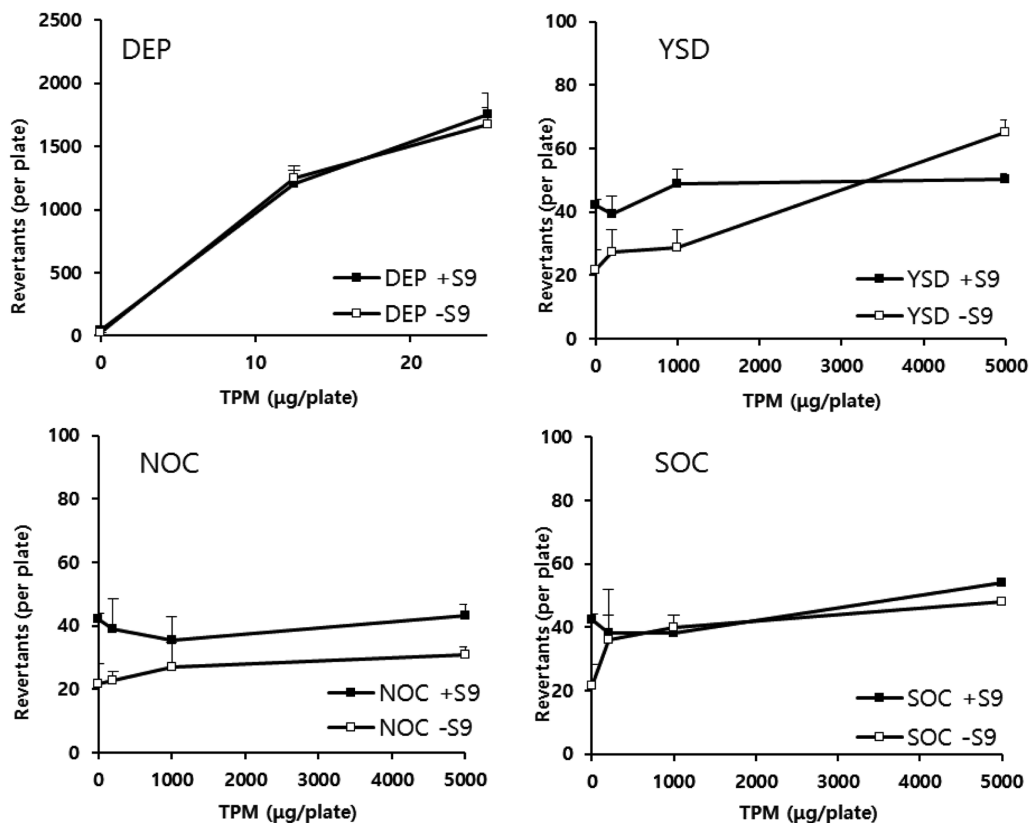


Fig. 2. Change in revertant colony numbers of DEPs, NOC ashes, SOC ashes, and YSD. *Salmonella typhimurium* TA 98 strains with and without S-9 metabolic activation systems were used to examine the potential of the air pollutant particles to induce mutation. The number of revertant colonies in DMSO-treated (0.1 mL/plate), 2AA-treated (0.5 μg/plate), and 4NQO-treated (0.5 μg/plate) groups was 34 ± 5, 579 ± 22, and 547 ± 24, respectively. DEP, diesel exhaust particles; NOC, natural organic combustion; SOC, synthetic organic combustion; YSD, yellow sand dust; DMSO, dimethyl sulfoxide; TPM, total particulate matter.

Table 1. Mutagenicity of total particulate matter (TPM) from four kinds of air pollutant particles

<i>Salmonella typhimurium</i> tester TA 98		
Sample	Specific activity with S9 (Revertants/mg)	Specific activity without S9 (Revertants/mg)
DEP	71,347 ± 2,183	66,289 ± 4,872
YSD	Nontoxic up to 5,000 µg/plate	
NOC ash	Nontoxic up to 5,000 µg/plate	
SOC ash	Nontoxic up to 5,000 µg/plate	
<i>Salmonella typhimurium</i> tester TA 100		
Sample	Specific activity with S9 (Revertants/mg)	Specific activity without S9 (Revertants/mg)
DEP	20,941 ± 1,119	23,739 ± 2,217
YSD	Nontoxic up to 5,000 µg/plate	
NOC ash	Nontoxic up to 5,000 µg/plate	
SOC ash	Nontoxic up to 5,000 µg/plate	

DEP, diesel exhaust particles; YSD, yellow sand dust; NOC, natural organic combustion; SOC, synthetic organic combustion.

system and, therefore, could not stimulate its growth (data not shown). NOC, SOC, and YSD particles did not promote the TA 98 strain growth even at high TPM concentrations. When the revertant colony number was measured following treatment with 1 mg TPM, the values for DEPs were 71,347 ± 2,183 and 66,289 ± 4,872 with and without the S-9 mix, respectively (Table 1). Consequently, DEPs and its metabolites exhibited a high mutagenic activity. However, none of the NOC, SOC, and YSD particles and their hepatic metabolites exhibited any mutagenic activity in the TA 98 strain culture system.

The changes in the number of revertant colonies at different concentrations of the four kinds of APPs are shown in Fig. 3. The characteristics of the TA 100 strain are useful for identifying base-pair substitution type mutations. In the TA 100 culture system, similar to the TA 98 strain, DEPs dose-dependently elevated the revertant colony number at low concentrations, and this number decreased at concentrations > 100 µg/plate of DEPs.

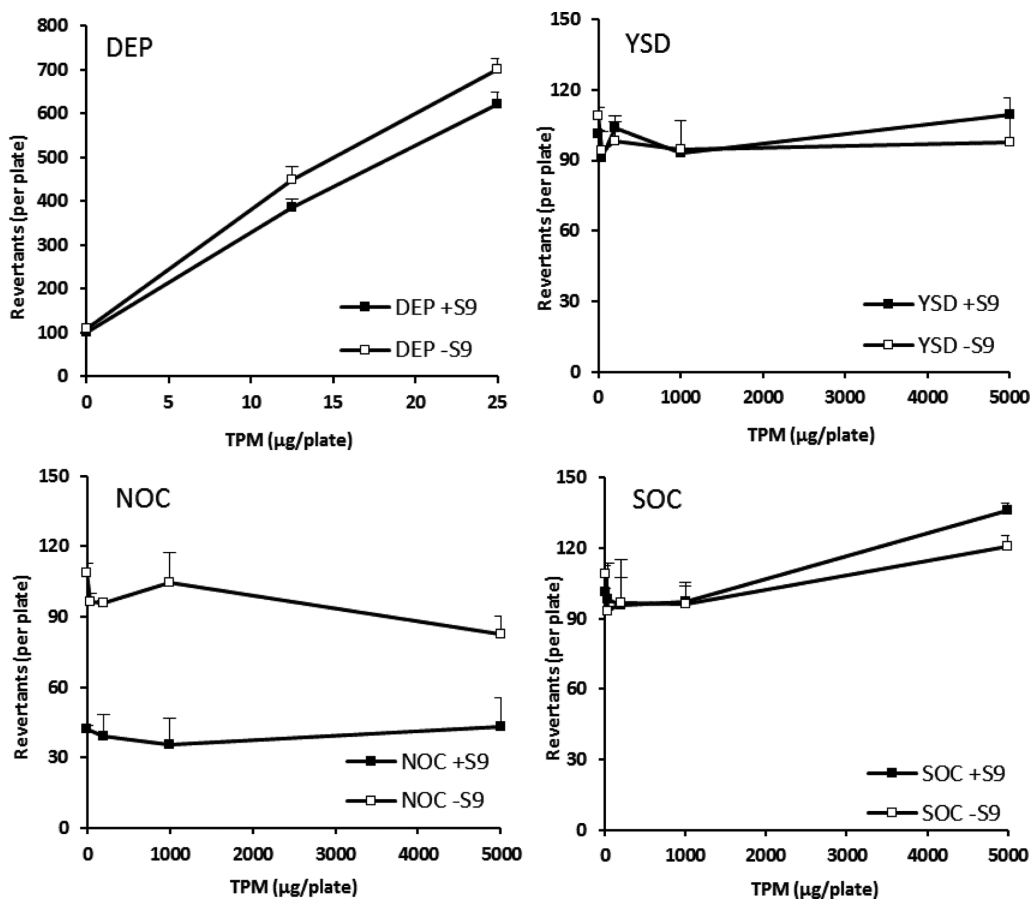


Fig. 3. Change in revertant colony numbers of DEPs, NOC ashes, SOC ashes, and YSD. *Salmonella typhimurium* TA 100 strain with and without S-9 metabolic activation system was used to examine the potential of the air pollutant particles to induce mutation. The number of revertant colonies in DMSO-treated (0.1 mL/plate), 2AA-treated (0.5 µg/plate), and SA-treated (1.0 µg/plate) groups was 123 ± 17, 1,745 ± 123, and 1,342 ± 98, respectively. DEP, diesel exhaust particles; NOC, natural organic combustion; SOC, synthetic organic combustion; YSD, yellow sand dust; DMSO, dimethyl sulfoxide.

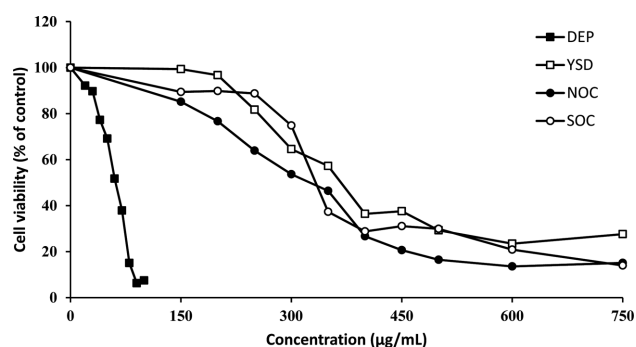


Fig. 4. Cytotoxicity of TPM of four kinds of air pollutant particles, DEPs, NOC ashes, SOC ashes, and YSD. CHO cells were treated with various concentrations of TPM (µg/mL) for 24 hr. Data are expressed mean ± SD. TPM, total particulate matter; DEP, diesel exhaust particles; NOC, natural organic combustion; SOC, synthetic organic combustion; YSD, yellow sand dust; SD, standard deviation.

However, NOC, SOC, and YSD particles in the TA 100 strain culture system did not increase the growth even at high doses, similar to that observed for the TA 98 strain. When the revertant colony number was measured on the basis of 1 mg TPM, the values for DEPs were 20,941 ± 61 and 23,739 ± 7 with and without the S-9 mix, respectively. Consequently, both DEPs and its metabolites had a high mutagenic effect. However, none the NOC, SOC, and YSD particles and their hepatic metabolites showed any mutagenic activity in the TA 100 strain culture system.

Cytotoxicity. Fig. 4 shows the change in cell viability following treatment of the CHO-K1 cell culture system with various concentrations of the four kinds of APPs. The TPM fractions of DEPs, NOC ashes, SOC ashes, and YSD were applied to CHO cells at a range associated with concentration-dependent responses. The viability of treated cell groups was calculated relative to that of the normal cells, which was considered 100%. DEPs decreased the cell viability precipitously at a low concentration, but NOC, SOC, and YSD particles decreased the viability gradually. Table 2 presents the change in cell viability following treatment with various concentrations and the EC₅₀ values that were

Table 2. Effective concentration (EC₅₀) values and relative cytotoxicity of four kinds of air pollutant particles

Samples	Cytotoxicity (EC ₅₀ , µg/mL)	Relative cytotoxicity (% of DEP)
DEP	58.2 ± 3.0	100
YSD	451.3 ± 36.6	12.9
NOC ash	339.0 ± 8.6	17.2
SOC ash	327.4 ± 33.3	17.8

DEP, diesel exhaust particles; YSD, yellow sand dust; NOC, natural organic combustion; SOC, synthetic organic combustion.

calculated to compare the relative cytotoxicity of the four FPs.

All four FP types showed dose-dependent cytotoxicity. The EC₅₀ values for DEPs, NOC ashes, SOC ashes, and YSD particles were 58.2 ± 3.0, 339.0 ± 8.6, 327.4 ± 33.3, and 451.3 ± 36.6, respectively. Therefore, we concluded that DEP exhibited the highest cytotoxicity of the four FP types, while NOC and SOC showed similar levels of cytotoxicity, which did not differ significantly. Furthermore, YSD showed the weakest cytotoxicity of all four FPs.

DISCUSSION

APPs are a complex mixture of organic and inorganic compounds and include a number of toxic compounds such as metals and PAHs. Combustion processes emit large quantities of particles into the atmosphere. Particles formed in combustion systems are classified into two categories. The first category, referred to as ash, comprises particles derived from noncombustible constituents. The second category consists of carbonaceous particles that are formed by pyrolysis of fuel molecules such as diesel, coal, and wood.

The purpose of this study was to investigate the physical characteristics and analyze the chemical elements in four kinds of APPs (DEPs, NOC ash, SOC ash, and YSD). In addition, we compared their *in vitro* toxicological activity, including mutagenicity and cell toxicity, to evaluate their relative contribution to human health effects.

Many previous studies have reported that the *in vitro* biological activity of ambient air particles showed different responses according to not only particle size, but also a different type of chemical element (24). The indirect- and direct-acting mutagenicity of DEPs, NOC ash, SOC ash, and YSD were determined using the Ames test on *S. typhimurium* TA 98 and 100 strains. DEP showed the highest specific activity in both strains, and its indirect mutagenicity was higher than its direct-acting mutagenicity in the TA 98 strain. However, in the TA 100 strain, the indirect mutagenicity was lower than the direct-acting mutagenicity. The aromatic amines, PAHs, and heterocyclic amines, in DEPs are known to induce mutations and carcinogenesis (25,26).

Cytotoxicity was found to be affected by the constituent, size, and exposure time of the test chemicals. The cytotoxicity results indicated the concentration-dependent effects of DEPs, NOC ash, SOC ash, and YSD.

Based on the EC₅₀ values of DEP, NOC, SOC, and YSD, we concluded that DEP showed the highest cytotoxicity of the four FP while NOC and SOC exhibited comparable levels of cytotoxicity with no significant difference. Furthermore, YSD showed the weakest cytotoxicity of all four FPs. Numerous previous studies have reported that higher content of metals or PAHs are associated with greater quantities of ROS produced in the particle-exposed cells (27,28).

Therefore, the high content of metals and PAHs in DEP could have mediated its acute cell cytotoxicity.

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