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In Vitro Evaluation of Atmospheric Particulate Matter and Sedimentation Particles Using Yeast Bioassay System

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Little information on the evaluation of airborne particulate matter (APM) and sedimentation particles from subway stations is available. The thermal metamorphism of train wheels generating toxic particles in subway stations is a possibility. In this study, the toxicity and physiological effects of particles from subway stations were evaluated using a yeast bioassay system. Estrogenic and antiestrogenic activities of APM in APM extracts from subway stations were determined. No estrogenic activity was found in the APM fractions and their S9-activated APM samples. Sedimentation dust samples also showed no estrogen activity. In contrast, extracts from sedimentation dust samples showed antiestrogen activity. Marked yeast toxicity was observed in the samples extracted from sedimentation dust. Potent yeast toxicity was also found in the S9-activated extracts from sedimentation dust. The results suggest that sedimentation dust from a semiclosed area of a subway system has antiestrogen activity, although both the origin and generation system of this activity are uncertain. These pollutants in sedimentation dust may change to a more toxic form *in vivo* by S9 activation.

1. Introduction

Airborne particulate matter (APM) of various sizes has different characteristics.[(1)] The chemical and physical properties of APM and the type of sedimentation particle responsible for these properties including the most harmful types of APM and sedimentation particle are not yet known. Thus, a reliable system for the assessment of human exposure to APM and the evaluation of subsequent adverse health effects is important to maintain optimal public health. Very fine particles resulting from thermal metamorphism may cause health problems. Also, suspended particulate matter (SPM), particularly fine particles smaller than 2.5 μm in aerodynamic diameter ($PM_{2.5}$), is a public health concern.[(2–5)] These $PM_{2.5}$ particles may cause the largest damage to the respiratory system because of their deposition in the lungs and respiratory tract and their ability to remain airborne for long periods. These fine particles float in air for a long time in semiclosed spaces, such as a subway station and an underground parking lot. SPM and sedimentation particles contain more carcinogens, mutagens, metals, and environmentally disruptive chemicals than larger particles suspended in air.[(6–9)] Chris-

ter and Per-Ake[10] and Sitzmann *et al.*[11] reported the measurements of APM in subways in London and Stockholm, respectively. The main air pollutants in urban areas of Japan and Europe are nitrogen dioxide and SPM, which are derived from vehicle emission.[12,13] Especially, it is reported that Diesel-powered vehicles emit some 30–100 times more particles than do gasoline-powered cars, and diesel exhaust particles comprise most of SPM in the urban atmosphere.[14] These air pollutants may cause lung cancer and chronic respiratory diseases. Maruyama *et al.*[15] reported the health risk of particulate matters in the air to humans using bioassay data and a mathematical model. Although it is unknown whether train wheels generate toxic particles, it is likely that the thermal metamorphism of train wheels occurs. Subway workers and people who use the subway regularly may be at high risk of APM exposure. The thermal metamorphism of train wheels generating toxic particles in subway stations is a possibility.

In this study, the toxicity and physiological effects of several particles in subway stations were evaluated using a yeast bioassay system. The estrogenic activity of the samples was evaluated by a two-hybrid assay using human estrogen receptor α (hERα). Compounds that enhanced metabolism were also identified and quantified using a rat S9 liver assay. Estrogen activity assay was performed using the extracts of the particles to evaluate the relationship between the estrogenic activity and the pollutant chemicals found in the extracts.

2. Materials and Methods

2.1 *Chemicals*

Napthalene-d8, acenaphthene-d10, phenanthrene-d10, fluoranthene-d10, and chrysene-d12 surrogate standards were purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). Estradiol-17β (β-E_2), 4-hydroxy-tamoxifen (4-OHT), tamoxifen, and glucose-6-phosphate (G-6-P) were purchased from Sigma (St. Louis, MO, USA). *t*-Stilbene (T-S) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). β-$NADP^+$ was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Rat liver S9 was obtained from Kikkoman Company (Tokyo, Japan). Zymolyase 20T was obtained from Seikagaku Co. (Tokyo, Japan). HEPES buffer and other chemicals and reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 *Sample collection and preparation*

Airborne particulates from subway platforms were collected using a personal cascade impact sampler (P.C.I. sampler: Tokyo Dyrec Co.) in Sapporo (March 17–18, 2004) and Fukuoka (May 3–4, 2003). The size cut-offs of the particulates for APM, SPM, and $PM_{2.5}$ are >10, 2.5–10, and <2.5 μm, respectively. Airborne particulates were collected on quartz fiber filters at a flow rate of 5 L/min. An amount of 1000 L of air was sampled at each subway station platform. Approximately 50 g of sedimentation particles was collected in a glass case. Airborne particulates collected using a filter were extracted by ultrasonication for 15 min with 20 ml of dichloromethane (DCM). After extraction, DCM was evaporated under a gentle nitrogen stream and the residue was dissolved in 500 μl of dimethyl sulfoxide (DMSO) or hexane. A 10-g sample of the sedimentation particles was extracted using the same procedure for extraction from the filters.

2.3 *Chemical analysis*

Samples were analyzed by electron impact gas chromatography-mass spectrometry (GC/MS), using a Hewlett Packard 6890 gas chromatograph interfaced to a 5973N Mass Selective Detector operated in the scan mode (Agilent Technologies). Identification of chemicals was carried out using the NIST98 database. The column

used for all experiments was a 30-m HP-5ms capillary column (5% phenyl methyl siloxsan; length, 30 m; i.d., 0.25 mm; film thickness, 0.25 μm; Hewlett Packard). The injection temperature was 280°C and the interface temperature was 280°C. The GC oven temperature was programmed to remain at 40°C for 1 min, to increase to 300°C at a rate of 10°C/min, and to maintain the final temperature for 12 min. Thermal desorption (TD) was performed using a Gerstel thermal desorption system (TDS) 2 equipped with a Gerstel TDS, an autosampler, and a Gerstel CIS 4 programmable temperature vaporization (PTV) inlet. GC/MS was performed using an Agilent 6890N gas chromatograph equipped with a 5973N mass-selective detector.

2.4 *TDS-CIS conditions*

The TDS 2 temperature was programmed to increase from 20°C (after a 1-min hold) to 280°C (held for 5 m) at 60°C/min. The desorbed compounds were cryofocused in the CIS 4 PTV inlet at –150°C. After desorption, the CIS 4 temperature was programmed to increase from –150 to 300°C (held for 10 m) at 12°C/s to inject the trapped compounds onto the analytical column. Injection was performed in the splitless mode.

2.5 *Estrogen agonist test using yeast two-hybrid assay*

β-E2 and T-S were used as positive controls for the –S9 and +S9 tests, respectively. The samples were dissolved in DMSO, dispensed into glass sample bottles in small doses, and preserved at –20°C.

The estrogenic activities of the extracts from the particulate matter and sedimentation particles were measured using a yeast two-hybrid assay, as described by Shiraishi *et al.*[16] Estrogenic activity also was measured with and without possible metabolic activations using a rat liver S9 preparation. Metabolic activation was performed after sample incubation with the rat liver S9 preparation (14 mg of $NADP^+$, 7 mg of G-6-P, 40 μl of HEPES buffer, 0.5 ml of rat liver S9 preparation, and 10 ml of Z buffer) for 1 h at 37°C. This test was performed with yeast cells (*Saccharomyces cervisiae* Y190) prepared by incorporating hERα, the expression plasmid of the coactivator TIF2, and a β-galactosidase expression reporter in a yeast two-hybrid assay.[16] Aliquots of sample solutions (20 μl) were incubated (30°C, 4 h) with yeast cells in a 96-well microplate (SUMILON, Sumitomo Bakelite, Japan) that had been preincubated (30°C, overnight) with a modified SD medium lacking tryptophan and leucine. A solution for inducing chemiluminesence and enzymatic digestion (Zymolyase 20T) was added followed by the addition of a light-emission accelerator solution. The chemiluminesence intensity produced by the released β-galactosidase was measured using a 96-well plate luminometer (Luminescencer-JNR AB2100, ATTO Bio-Instrument, Tokyo, Japan). Estrogen agonist activity was recorded as EC×10, which is defined as the concentration of the test solution producing a chemiluminescence signal 10×blank control. The inverse of the EC×10 values obtained for β-E2 and T-S was set to 100. Similar procedures for calculating the β-E2 relative activity were used for the other samples.

2.6 *Estrogen antagonist test using yeast two-hybrid assay*

Estrogen antagonist activity was measured in a yeast two-hybrid assay using yeast cells (Y190) and hERα. Aliquots of the test solutions, including 300 pM β-E2, were incubated (30°C, 4 h) with yeast cells that had been preincubated (30°C, overnight) in a modified SD medium (lacking tryptophan and leucine). A solution for inducing chemiluminescence and enzymatic digestion (Zymolyase 20T) was added; this was followed by the addition of a light-emission accelerator solution. The chemiluminescence intensity was measured using a luminometer (Luminescencer-JNR AB2100, ATTO Bio-Instrument, Tokyo, Japan). Estrogen antagonist activity was recorded as IC×50, which is defined as the concentration of the test solution produc-

ing a chemiluminescent signal 50×blank control. The inverse of the IC×50 values obtained for 4-hydroxytamoxifen and tamoxifen was set to 100.

2.7 *Yeast toxicity test*

The toxicity of the test samples toward yeast cells in the two-hybrid assay was determined using the yeast cell strain YTOX,[17] which continuously produces β-galactosidase. A decrease in chemiluminescence intensity indicates yeast cell damage.

3. Results and Discussion

Samples were collected at subway stations A and B (Sapporo) and C (Fukuoka). The typical chemical components of the airborne fractions of APM, SPM, and $PM_{2.5}$ were identified by a search of the MS spectrum library (NIST 98). The number of chemicals from the typical TIC patterns of the airborne fractions of APM, SPM, and $PM_{2.5}$ from three subway stations are shown in Table 1. The samples identified from station A included 170 types of APM, 145 types of SPM, and 161 types of $PM_{2.5}$. From station B, 148 types of APM, 118 types of SPM, and 161 types of $PM_{2.5}$ were identified. From station C, 70 types of APM, 65 types of SPM, and 125 types of $PM_{2.5}$ were identified. The main components of the chemicals were 2-ethyl-hexanol, octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5), dodecamethylcyclohexasiloxane (D6), diethyl phthalate (DEP), dibutyl phthalate (DBP), and diethyl-hexyl phthalate (DEHP). The sedimentation particles from station A contained 91 types of chemical components identified from the NIST 98, whereas particles from stations B and C contained 61 and 94 types, respectively. In a previous study, it was demonstrated that several phthalate esters have agonist or antagonist activities for human breast cancer estrogen-sensitive MCF-7 cells.[18] In a proliferation assay using MCF-7 cells to assess estrogenic activity, the value of DEHP was estimated to be 10^{-3} M.[18] Additionally, in an antiestrogen assay, the values of DEP and DBP were estimated to be 10^{-3} M.[18] Le Vier and Boley evaluated the antigonadotropic activity of cyclosiloxanes in male rats, and the hormone activity was decreased at 24 h by cyclosiloxane.[19] In this study, we found DEHP,DEP, DBP, D4, D5, and D6 in subway air and sedimentation particles. These compounds may have adverse effects on human health.

In this study, estrogen and antiestrogen activities were determined in airborne particulates from APM, SPM, and $PM_{2.5}$ fractions. The toxicity of the sedimenta-

Table 1
Typical TIC patterns of APM, SPM, $PM_{2.5}$, and sedimentation particles listed in order of chemical compound number.

Site	APM	SPM	$PM_{2.5}$	Sedimentation particle	Detected main chemicals
A	170	145	161	91	BT, D5, D6, BHT, DEP DBP, DEHP, HHCB
B	148	118	161	61	D5, D6, BHT, DEP, DBP, DEHP, HHCB
C	70	665	125	94	ET, D5, D6, DBP, DEHP, HHCB

Abbreviations: 2-butoxyethanol (BT), 2-ethyl-hexanol (ET), Dibutylhydroxytoluene (BHT), and 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB)

tion particles toward yeast was also determined. No estrogenic agonist or antagonist activity was found for the APM, SPM, and $PM_{2.5}$ samples from the three subway stations (data not shown). These samples did not show any estrogen agonist or antagonist activity after activation by S9, which is activated by the supernatant of the 9000-g fraction of the rat liver preparation. In contrast, the extract from the sedimentation particles (–S9-SP) of the subway stations A and B showed antiestrogenic activity in the yeast assay (Figs. 1 and 2). On the other hand, the S9-activated extract sedimentation particles (+S9-SP) from stations A and B showed a 10- to 300-fold higher yeast toxicity than that of the diluted samples in a dose-dependent manner. Besides samples A and B showing no estrogenic activity, the extracted samples (500×dilution) and these samples showed weak antiestrogenic activity. Although it is considered that the components of sedimentation particles of subway stations A and B (Table 1) are similar, a significant difference was not noted on yeast toxicity between subway stations A and B after activation by S9 treatment. The yeast toxicity of +S9-SP from 500×dilution extracted samples in subway station B is not significantly different as compared with that of –S9-SP.

A significant effect to the yeast system was not detected in the sedimentation particle sample from station C. A decrease in yeast toxicity level by dilution may enable the detection of physical responses by S9-SP. Thus, +S9-SP may have antiestrogenic activity if the toxicity is not considered. Although antiestrogenic activity was found in the sedimentation particle extracts from subway stations A and B, the

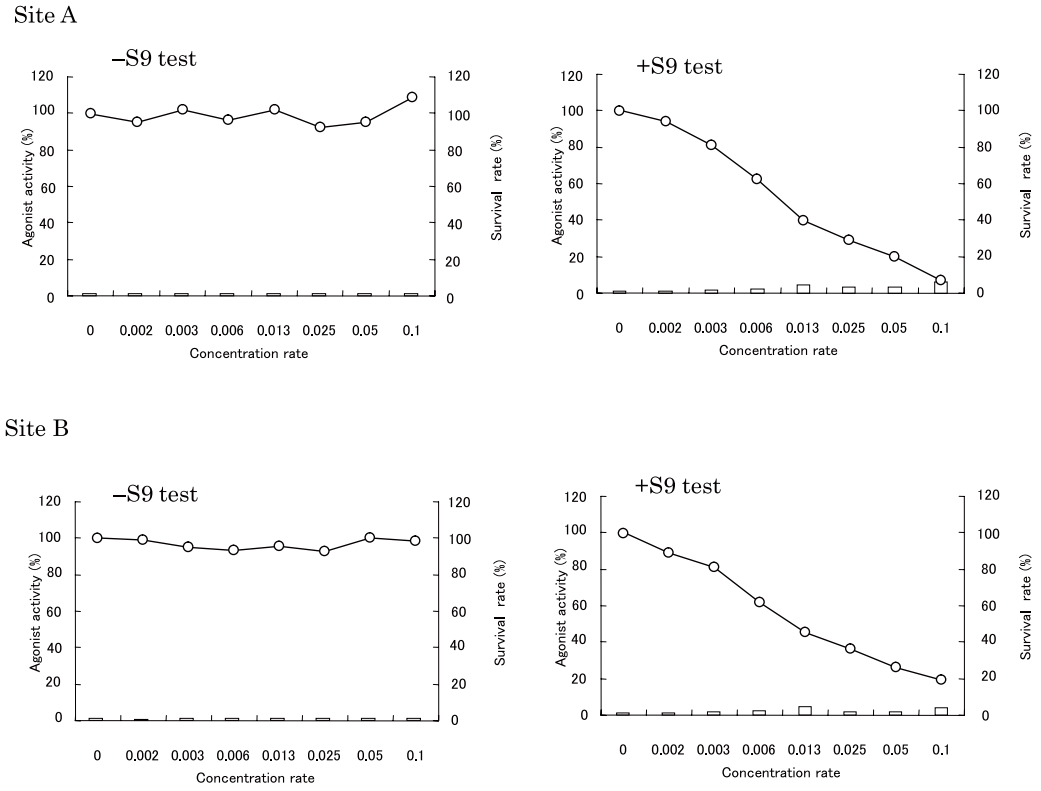

Fig. 1. Dose-response curves of estrogenic activity (white bar) and toxicity (white symbol) for sedimentation particles from subway stations A (upper) and B (lower) using yeast two-hybrid assay. The results of the samples before and after rat S9 treatment are shown in the left and right figures, respectively. The values for estrogenic agonist activity are represented as activity (%) of chemiluminescence intensity, and the values for survival rate are represented as residual rate (%) of the chemiluminescence intensity of β-galactosidase.

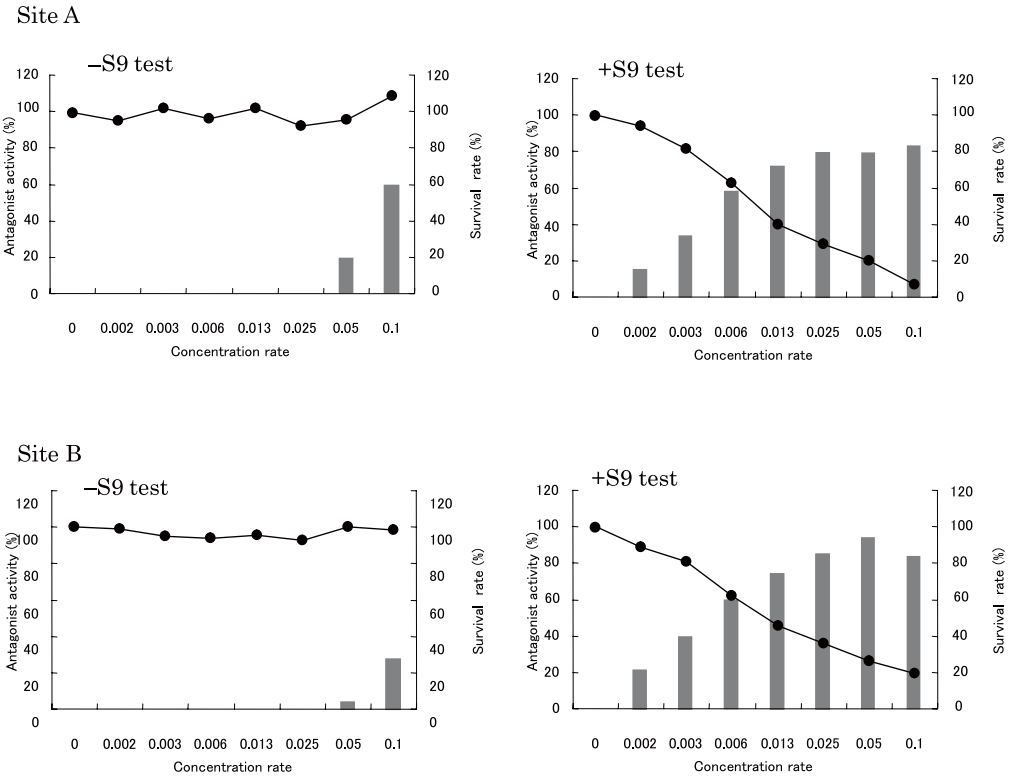

Fig. 2. Dose-response curves of antiestrogenic activity (black bar) and toxicity (black symbol) for sedimentation particles from subway stations A (upper) and B (lower) using yeast two-hybrid assay. The results of the samples before and after rat S9 treatment are shown in the left and right figures, respectively. The values for estrogenic antagonist activity are represented as inhibition (%) of chemiluminescence intensity, and the values for survival rate are represented as residual rate (%) of the chemiluminescence intensity of β-galactosidase.

concentrated extracts of APM, SPM, and $PM_{2.5}$ from subway stations A and B may show antiestrogenic activity if they are evaluated by more sensitive methods. Sedimentation particles and gas from several urban and rural areas have been assessed for their estrogenic activity using an estrogen receptor, and all samples were found to show estrogenic activity.[20] The estrogenic activity range of particulate samples was 0.3–18.0 m^3 (20% of 10^{-10} estradiol) as determined by an ER reporter gene assay.[20] In this study, none of the particulate and sedimentation samples showed estrogenic activity; thus, it is considered that insufficient amount of samples was collected from air.

Granular or powdered SPM and $PM_{2.5}$ particles in air may be generated from train wheels by thermal metamorphism and dispersed as dust. The sedimentation particles come from airborne particles. It is important to evaluate the toxicity of +S9-SP, which is activated by the supernatant of the 9000-g fraction of the rat liver preparation. Toxic chemicals may be produced in the lungs and liver after inhalation. Thus, the health of subway workers and regular passengers may be at risk because of +S9-SP toxicity. The subway system in Sapporo (subway stations A and B) is different from the other subway systems in Fukuoka. The subway trains in Sapporo have a rubber-tire wheel system. Although it is unclear whether a rubber-tire wheel generates toxic particles, thermal metamorphism from a rubber-tire wheel may be taken into consideration.

Particulates such as SPM are important to health not only because they persist in the atmosphere longer than larger particles, but also because they are sufficiently inhaled and that these particulates penetrate deep into the respiratory tract. APM, including sedimentation particulates containing toxic chemicals, may induce various toxic effects. The possibility of an airborne particle being inhaled depends on its aerodynamic diameter, the velocity of the surrounding air, and the person's respiration rate. Studies of long-term exposure to airborne particles including sedimentation particulates generated from a rubber-tire wheel suggest that exposure to these particles may cause increases in mortality rate and the risk of chronic respiratory illness, including the development of various types of cancer. Future studies are needed to confirm the physiological effects of +S9-SP generated from rubber-tire wheels.

4. Conclusions

The typical chemical components of the airborne fractions of APM, SPM, $PM_{2.5}$, and sedimentation particles were identified by a search of the MS spectrum library. The estrogenic and antiestrogenic activities of extracts from fractionated particle samples from subway stations were evaluated. Estrogenic activity was not observed in the samples by a yeast two-hybrid assay, regardless of activation by S9 treatment. Extracts from sedimentation particles also showed no estrogenic activity; however, the sedimentation particle samples obtained from subway stations have antiestrogenic activity, although they showed yeast toxicity after activation by S9 treatment. Airborne particulates generated from rubber-tire wheels need to be evaluated for their antiestrogenic activity with consideration of their physiological toxic effects.

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