J. Vet. Sci. (2004), **5**(1), 11–18

JOURNAL OF
Veterinary
Science

Comparative study of PM2.5 - and PM10 - induced oxidative stress in rat lung epithelial cells

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Accurate estimation of the exposure-response relationship between ambient urban particulate matters (PM) and public health is important for regulatory perspective of ambient urban particulate matters (PM). Ambient PM contains various transition metals and organic compounds. PM10 (aerodynamic diameter less than 10 μm) is known to induce diverse diseases such as chronic cough, bronchitis, chest illness, *etc*. However, recent evaluation of PM2.5 (aerodynamic diameter less than 2.5 μm) against health outcomes has suggested that the fine particles may be more closely associated with adverse respiratory health effects than particles of larger size. This study was performed to evaluate PM2.5-induced oxidative stress in rat lung epithelial cell in order to provide basic data for the risk assessment of PM2.5. PM2.5 showed higher cytotoxicity than PM10. Also, PM 2.5 induced more malondialdehyde (MDA) formation than PM10. In Hoechst 33258 dye staining and DNA fragmentation assay, apopotic changes were clearly detected in PM2.5 treated cells in compared to PM10. Expression of catalase mRNA was increased by PM2.5 rather than PM10. PM2.5 induced higher Mth1 mRNA than PM10. In pBR322 DNA treated with PM2.5, production of single strand breakage of DNA was higher than that of PM10. In Western blot analysis, PM2.5 induced more Nrf-2 protein, associated with diverse transcriptional and anti-oxidative stress enzymes, compared to PM10. Our data suggest that PM2.5 rather than PM10 may be responsible for PM-induced toxicity. Additional efforts are needed to establish the environmental standard of PM2.5.

Key words: particulate matter 2.5 (PM2.5), particulate matter 10 (PM10), rat lung epithelial cell

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Introduction

Air pollutants have been recognized as a major problem for human health. Airborne particulate matters (PMs) are associated with pulmonary diseases including cancer [1,6,21,22]. PMs are known to cause DNA, protein damage and apoptosis through mitochondria-regulated death pathway [3,17]. Also, there is evidence that metals in PMs can induce DNA and protein damage [16]. However, the precise mechanisms are not clear.

PMs can be classified by size. PM10, the coarse fraction, is particles having mass median aerodynamic diameter <10 μm and PM2.5, the fine fraction, is smaller than 2.5 μm. However, the similarity of chemical components and physical characteristic between PM10 and PM2.5 is little [19]. In the chemical components, the coarse fraction (PM10, particles >2.5 μm) is dominated by natural sources (fugitive and resuspended dust, biological materials such as pollen, bacteria), while the fine fraction (PM2.5, particle <2.5 μm) is dominated by anthropogenic emissions [12]. In the physical characteristic, PM2.5 has the ability to reach the lower regions of the respiratory tract than PM10 does. PM10 was associated with increased frequencies of chronic cough, bronchitis, chest illness and mortality [21]. Suspended PM10 is complex aggregates of inorganic material, salts (nitrates, sulfates), organic material [2]. In 1987, U.S. Environmental Protection agency (U.S.EPA) replaced the earlier total suspended particulate (TSP) air quality standard with PM10 standard. Our government also has controlled the air quality on the standard of PM10 until now. However, recent studies suggested that PM2.5 might cause serious adverse health effects. As a result, U.S.EPA strengthened its health protection standards for PM by adding an indicator for even "fine" particles (PM2.5). In this trend, our government is trying to update on ongoing litigation over PM2.5 standard. However, relative less information of PM2.5 is present to evaluate the

risk of PM2.5. Therefore, this study was performed to compare *in vitro* toxicity of PM2.5 and PM10 collected in urban area of Seoul, Korea.

Materials and Methods

Collection of PMs

Airborne PMs were collected using high-volume air samplers (Andersen, USA). Based on the principle of virtual impaction, particles are separated into a fine mode (<2.5 μm) and a coarse mode (<10 μm). Two fractions of particles were collected onto two separate filters: one filter containing PM10 and the other filter containing PM2.5.

Preparation and extraction of filters

Teflon filters were baked for 2 h at 100°C and transferred into 50 ml conical tube with the particles facing 0.1 × PBS. Extraction of the PM*s* took place in an ultrasonic bath three times for 20 minutes. The extracts were lyophilized overnight at −80°C in a vacuum. The pellet was collected and removed the biological species such as pollen and endotoxin. Collected PM*s* were weighted and resuspended in PBS solution. Resuspension took place in an ultrasonic bath for 30 minutes. And stock solution was stored at −20°C until use.

Cell culture

Rat lung epithelial cell, rat type II epithelial origin, was obtained from ATCC (Manassas, USA) and cultured Ham's F-12 media (Gibco) containing 2 mM L-glutamine supplemented with 0.01 mg/ml bovine pituitary extract (Gibco, USA), 0.005 mg/ml insulin (Sigma, USA), 2.5 ng/ml IGF (Sigma), 0.025 mg/ml trasferrin (Sigma, USA), and 10% FBS under 5% CO_2, 37°C and 100% humidified condition.

Cytotoxicity test

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded in 96-well tissue culture dishes with 50,000 cells per well. They were cultured for 24 h. The medium was then replaced. After rat lung epithelial cells were treated with various concentrations of PM2.5 and PM10 stock solution, the cytotoxicity was measured by MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay. Twenty-four hours after the treatment of various concentrations of PMs, 10 μl of MTT (Sigma) solution (5 mg/ml) was added, and incubated for 4 hours at 37°C. Cells were lysed in 100 μl of dimethylsulfoxide (DMSO, Sigma) and absorbance was quantified using ELISA reader (BioRad, USA).

Measurement of malondialdehyde (MDA)

Malondialdehyde was quantitated using spectrophotometer method [16]. After removal of media, the membranes were solubilized in 400 μl of 8% SDS (Sigma), and added 25 μl of 4% butylatedhydroxy toluene (Sigma) in ethanol, 500 μl of 10% phosphotungstinic acid (Sigma) in 0.5 M sulfuric acid in a serial manner. After addition of 250 μl of 0.7% thiobarbituric acid (Sigma), the tubes were placed in boiling bath for 50 min. Then, 1 ml of 1-butanol (Sigma) were added, and the tubes were centrifuged and the supernatant containing thiobarbituric acid reactants (TBARs) was collected to measure the absorbance at 535 nm. TBARs were quantitated using a standard curve prepared with a 1 mM solution of tetrahydroxypropane (Sigma) hydrolyzed in 1% sulfuric acid.

Hoechst 33258 dye staining

The cells were fixed with a 10% formalin phosphate buffer solution (pH 7.4) for 5 minutes at room temperature. After washing with distilled water, cells were stained with Hoechst 33258 (Sigma) at the concentration of 8 μg/ml for 5 minutes. The cells were washed again with distilled water. The fluorescence was measured using a fluorescence microscope (Zeiss, Germany) with the excitation and emission wavelength at 340 and 510 nm, respectively.

Analysis of DNA fragmentation

The DNA sample in a loading buffer [50 mM Tris, 10 mM EDTA, 1% (W/V) low melting point agarose, 0.25% (W/V) bromophenol] was loaded onto per solidified, 1.8% (W/V) agarose gel containing 0.1 g/ml ethidium bromide. A 100bp DNA ladder (Promega) standard marker was also loaded to help verify the size of the products. Agarose gels were run at 50 V for 90 minutes in 1 × TBE buffer (108 g Tris + 55 g boric acid + 40 ml of 0.5 M EDTA, made up to 1 L with water). Finally, gels were visualized and photographed by computerized UV densitometer (BioRad).

Single strand breakage assay (SSBs assay) using pBR322 DNA

To measure PM-induced oxidative DNA damage, single strand DNA breakage assay was performed according to Nampalli *et al.*'s method [20]. Briefly, 2 μg of pBR322 DNA (TaKaRa, Japan) was suspended in 50 μl of TE buffer (pH 7.4) containing PM2.5 and PM10 stock, and then incubated at 37°C for 1 day. The exposed DNA samples were examined for the formation of SSBs. Electrophoretic separation of form I and form II was achieved on 0.7% agarose gel using 0.5 × TBE buffer at 50 v for 1 hr. DNA bands were stained with ethidium bromide and quantified with a UV densitometer (BioRad).

Reverse transcription-polymerase chain reaction (RT-PCR) of mRNA of repair enzymes

To study the steady-state mRNA levels of the genes that could be induced by PMs, rat lung epithelial cells were treated with different concentrations of PMs for 1 day, and

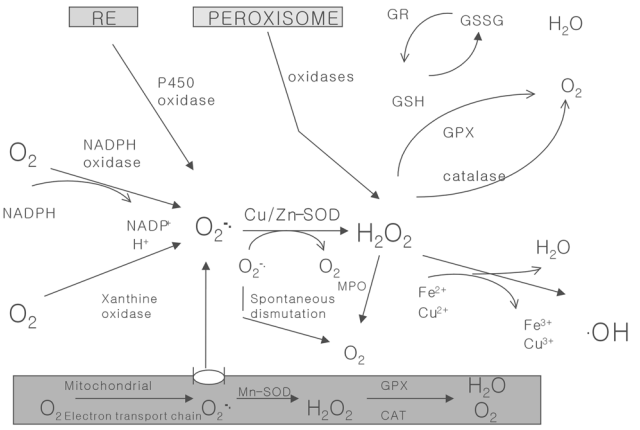

Fig. 1. The generation of reactive oxygen species (ROS) and main defense mechanisms against damage produced by reactive oxygen species (adapted from Mates *et al*., 2000)

total cellular RNA was isolated with TRI reagent (Sigma) according to manufacturer's protocol. The RNA was quantified by measuring its absorbance at 260 nm and 280 nm. Its integrity was confirmed by visualization of the ethidium bromide stained 28S and 18S ribosomal RNA bands in 1% agarose gel. mRNA levels were shown using Superscript II reverse transcriptase kit (Gibco) following manufacturer's manual. The number of amplification cycles was previously determined to keep amplification in the linear range to avoid the 'plateau effect' associated with increased number of PCR cycles. The RT-PCR primers were synthesized by Bioneer (Taejon, Korea) according to the Genbank (www.ncbi.nlm.nih.org) sequences. Mn-SOD (782 bp): sense 5'-GATGTGTGGAGCACGCTTACT-3' and antisense 5'-CACAATGTCACTCCTCTCCGAATTA-3', Catalase (763 bp): sense 5'-TTACTTTCTTGTTCAGCGAC CGA-3' and antisense 5'-C ACCTTCGTATAGAATGTCCG CA-3', Cu/Zn-SOD (541 bp): sense 5'-AGGATTAACTGA AGGCGAGCATG-3' and antisense 5'-GCCCAAGTCATC TTGTTTCTCGT-3', MTH1 (169 bp): sense 5'-AGCCTCA GCGAGTCTCCTG-3' and antisense 5'-GATCTGGGCCC ACCTTGTGC-3', beta-actin (273 bp): sense 5'-CCTGACC CTGAAGTACCCCA-3' and antisense 5'-CGTCATGCAGC TCATAGCTC-3'. After synthesis of first strand cDNA, PCR amplification program consisted denaturation at 94°C for 30 sec, annealing at 55-61°C for 45 sec, extension at 72°C for 1 min, 25-30 cycles, final extension at 68°C for 5 min. PCR products were electrophoresed in 2% agarose gel with ethidium bromide.

Western blot analysis

Cell pellet was washed PBS twice, and resuspended with lysis buffer (50 mM Tris at pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% igapel 630 (Sigma), and 0.5% edoxychoate) and centrifuged at 12,000 g for 1 hours. Equal amounts of protein were separated on an SDS-12% polyacrylamide gel and transferred to nitrocellulose membranes (Hybond ECL; Amersham, USA). The blots were blocked for 2 h at room temperature with skim milk in Tris-buffered saline containing 0.05% Tween-20. The membrane was incubated for 3hours at room temperature with Nrf-2 antibody. Detection of immunoreactive proteins was performed with the ECL Western blotting detection system (Amersham).

Statistical analysis

Data were expressed as mean ± SD. For comparison of means, Student's *t*-test was performed using SPSS 9.0 (SPSS Inc., USA) statistical package.

Results

Chemical-components of PMs

The ratio of heavy metals in PMs was more abundant in PM2.5 than PM10 (Fig. 2). PM2.5 contains more divese heavy metals than PM10.

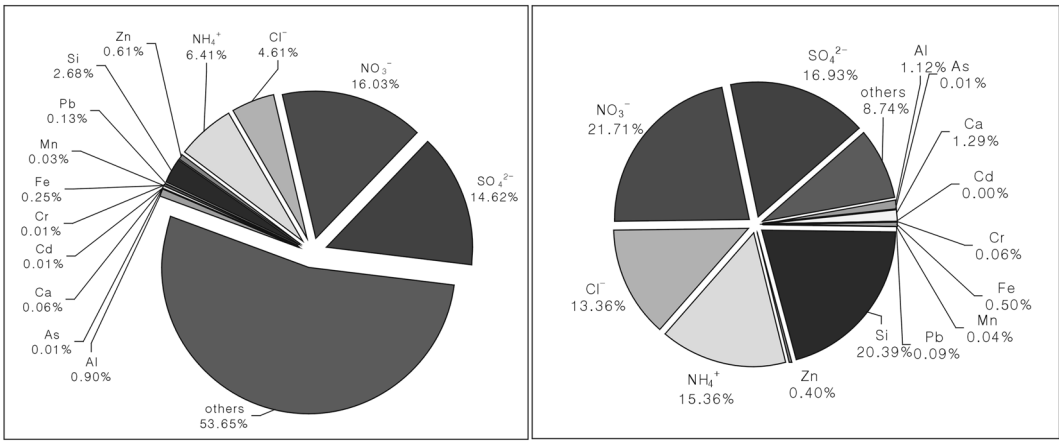

Fig. 2. The proportion of chemical components in PM2.5 and PM10. PM2.5 and PM10 used in this study (The left shows chemical components of PM2.5 the right shows chemical components of PM10).

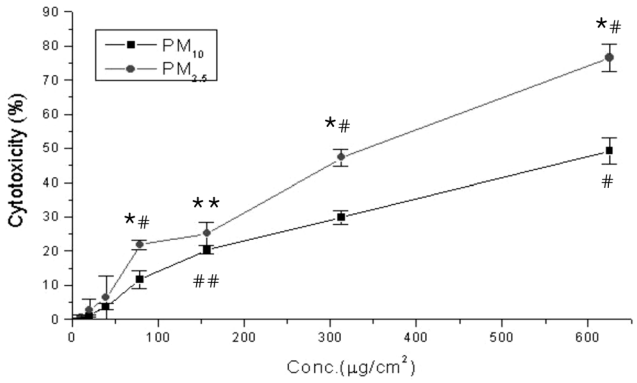

Fig. 3. Dosage response curve of stock solution derived from PM2.5 and PM10 assessed by MTT assay in rat lung epithelial cell. Cells were treated with PMs for 1 day. Values represent mean SD (n=5). *: Significantly different from PM10-treated group at same dose ($P<0.05$). **: Significantly different from PM10-treated group at same dose ($P<0.01$). #: Significantly different from lower dose-treated group ($P<0.05$).

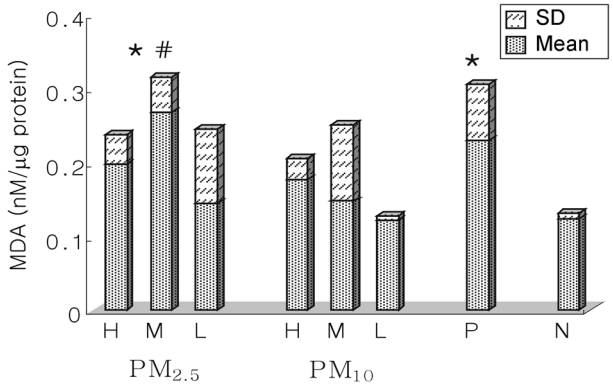

Fig. 4. Effects of PM2.5 and PM10 on the lipid peroxidation by oxidative stress. Cells were treated PMs for 1 day (PM2.5 H: 5 μg/cm^2, PM2.5 M: 1 μg/cm^2, PM2.5 L: 0.2 μg/cm^2, PM10 H: 5 μg/cm^2, PM10 M: 1 μg/cm^2, PM10 L: 0.2 μg/cm^2, P: H_20_2 300 nM, N: untreated cell). Values represent mean SD (n=3). The level of MDA induced by PM2.5 was higher than PM10. *: Significantly different from PM10-treated group at same dose ($P<0.05$). #: Significantly different from no treated group ($P<0.05$).

Cytotoxicity test by MTT assay

MTT assay was performed with various concentrations of PM2.5 and PM10 in rat lung epithelial cells (Fig. 3). As shown in Figure 3, PM2.5 showed more cytotoxicity than PM10 did in a concentration-dependent manner.

Measurement of lipid peroxidation through MDA

The production of malondialdehyde in both PM2.5-treated and PM10-treated cells were higher than that of untreated cells (Fig. 4). Generally, the level of MDA by PM2.5 was higher than that of PM10, with significantly high level of medium PM.

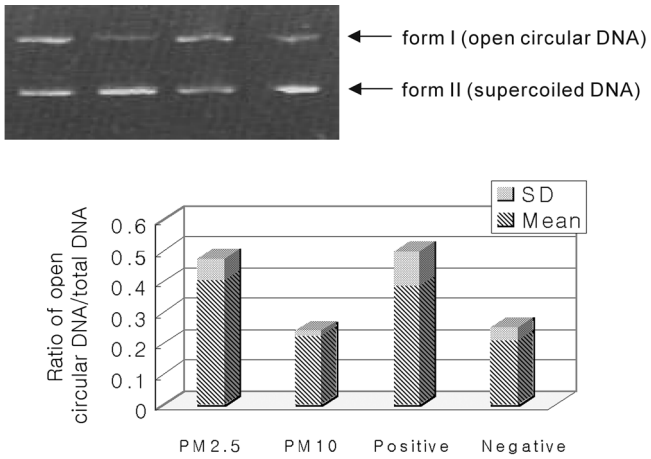

Fig. 5. Reactions by using strand breaks in plasmid pBR322. DNA is shown as reduction of form II (open circular DNA). The graph shows the ratio of open circular DNA/total DNA. See the materials and methods for detailed instruction. Values represent mean ± SD (n=3). The ratio of changed form II/total plasmid DNA (form I+form II) in PM2.5-treated DNA was significantly increased compared to both untreated-DNA and PM10-treated DNA. *: Significantly different from negative group ($P<0.05$). #: Significantly different from PM10-treated group at same dose ($P<0.05$).

SSBs assay using pBR322 DNA

The single strand breakage assay using pBR322 DNA was performed, the results were shown in Fig. 5 and added the graph that explained the ratio of changed form II / total plasmids DNA (form I + form II). The level of single strand breakage of pBR322 DNA by 25 μg/ml of PM2.5 was highly observed than 25 μg/ml of PM10. The results showed that the ratio of changed form II / total plasmid DNA (form I + form II) in PM2.5-treated DNA was significantly increased compared to both untreated DNA and PM10-treated DNA ($P<0.05$) (Fig. 5).

Hoechst 33258 dye staining Assay

As shown in Fig. 6, condensed nucleus stained with Hoechst 33258 fluorescence dye in cells treated 25 μg/cm^2 of PM2.5, shown as arrow, was much clearly detected than PM10.

Analysis of DNA fragmentation

To detect the apoptosis pattern induced by three concentration of PM2.5 and PM10 (25, 5, 1 μg/cm^2), DNA fragmentation assay was performed. In the cell treated with PM2.5, DNA laddering pattern was clearly found compared to PM10 treated cells (Fig. 7).

RT-PCR analysis of the gene expression of repair enzymes

To examine the effect of PM2.5 and PM10 to oxidative stress repair enzyme, we used RT-PCR analysis. Figure 8 shows the expression level of catalase mRNA induced by

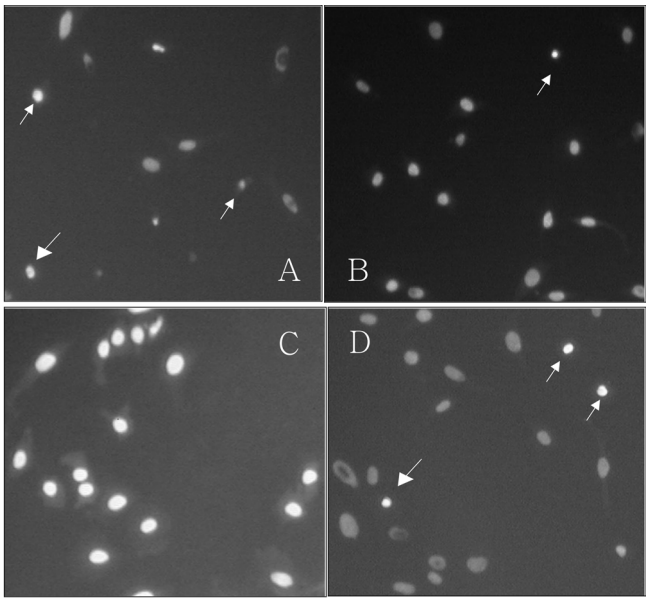

Fig. 6. The apoptotic pattern of rat lung epithelial cells stained with Hoechst 33258 dye. The arrows indicate nuclear condensation and apoptotic change. (A: PM2.5 25 μg/cm^2, B: PM10 25 μg/cm^2, C: untreated cell, D: H_2O_2 300 nM). Condensed nucleus stained with Hoechst 33258 fluorescence dye in the cells treated with 25 μg/cm^2 of PM2.5 was much clearly detected than PM10.

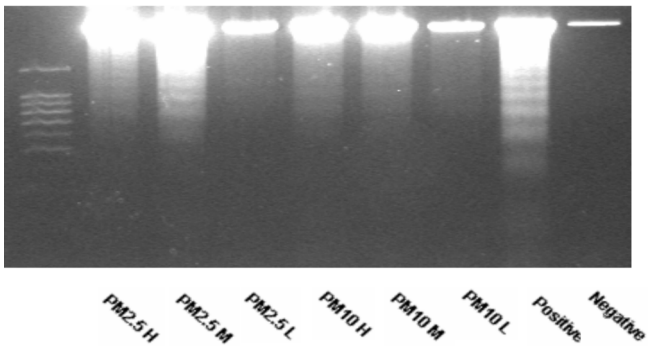

Fig. 7. DNA fragmentation assay. Genomic DNA was extracted from cells treated PMs. (PM2.5 high: 25 μg/cm^2, PM2.5 mid: 5 μg/cm^2, PM2.5 low: 1 μg/cm^2, PM10 high: 20 μg/cm^2, PM10 mid: 5 μg/cm^2, PM10 low: 1 μg/cm^2, positive: H_20_2 300 nM, negative: untreated cell, Marker: 100 bp DNA ladder marker). In the cell treated with PM2.5, DNA laddering pattern was seriously found compared to PM10 treated cell. In the sample treated with PM2.5 5 μg/cm^2, DNA Fragmentation pattern was the most seriously observed.

three concentration of PM2.5 and PM10 (5, 1, 0.2 μg/cm^2) for 1day. The level of catalase mRNA induced by PM2.5 was gradually decreased in concentration-dependent manner. In contrast, there was no remarkable change in PM10-treated group. As shown in Fig. 9 and 10, no distinct changes were detected in the expression of Mn-SOD mRNA

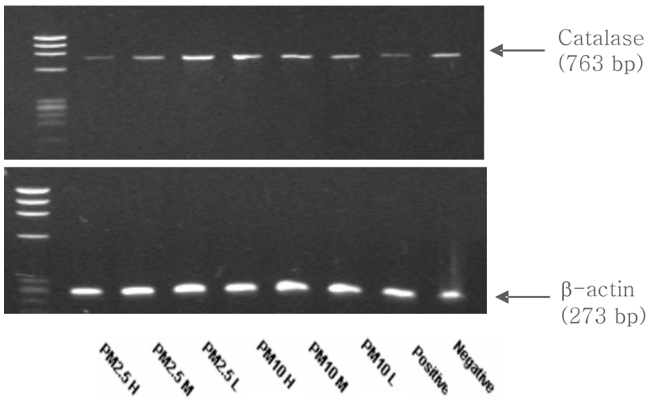

Fig. 8. RT-PCR analysis of catalase mRNA. The level of catalase mRNA induced by PM2.5 was decreased with concentration-dependent manner. β-actin was used for co-amplification (internal standard). H: 5 μg/cm^2, M: 1 μg/cm^2, L: 0.2 μg/cm^2, Positive: sample treated with H_2O_2 300 nM, negative: not treated sample.

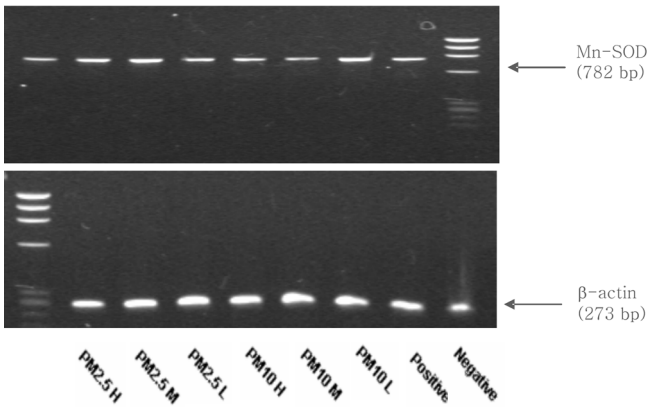

Fig. 9. RT-PCR analysis of Mn-SOD mRNA. No distinct changes were detected in the expression of Mn-SOD mRNA induced by PM2.5 and PM10. β-actin was used for co-amplification (internal standard). H: 5 μg/cm^2, M: 1 μg/cm^2, L: 0.2 μg/cm^2, Positive: sample treated with H_2O_2 300 nM, Negative: untreated cell.

and Cu/Zn-SOD mRNA. In the cells treated with 5 μg/cm^2 of PM2.5 and PM10, the level of Mth1 gene, DNA repair enzyme, was slightly highly expressed than others. Generally, expression level of Mth1 mRNA induced by PM2.5 was higher than PM10.

Western blot analysis of Nrf-2

No remarkable change of expression level in Nrf-2, proteins related with many transcriptional and anti-oxidative stress enzyme, was observed in the groups of three concentration of PM2.5 and PM10 (5, 1, 0.2 μg/cm^2). However, Nrf-2 protein induced by PM2.5 was higher than PM10 (Fig. 13).

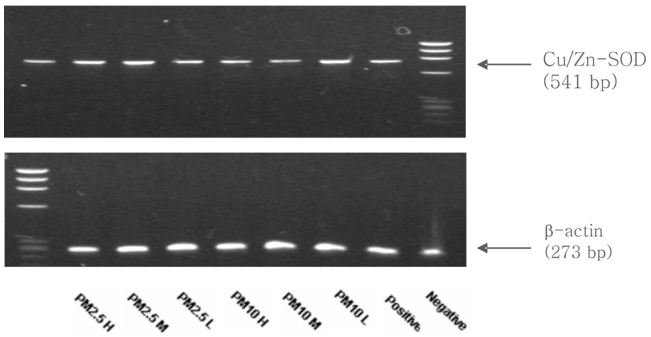

Fig. 10. RT-PCR analysis of Cu/Zn-SOD mRNA. No distinct changes were detected in the expression of Mn-SOD mRNA induced by PM2.5, and PM10. β-actin was used for co-amplification (internal standard). H: 5 μg/cm^2, M: 1 μg/cm^2, L: 0.2 μg/cm^2, Positive: sample treated with H_2O_2 300 nM, Negative: untreated cells.

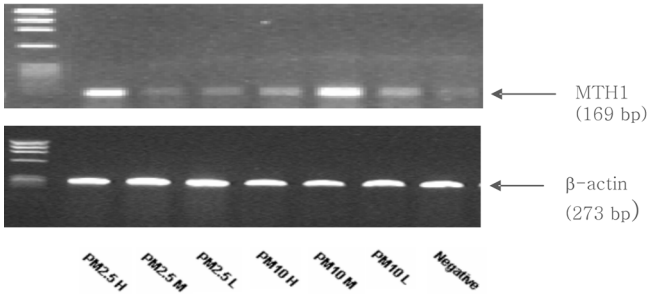

Fig. 11. RT-PCR analysis of MTH1 mRNA. MTH1 mRNA induced by PM2.5 was higher than PM10. β-actin was used for co-amplification (internal standard). H: 5 μg/cm^2, M: 1 μg/cm^2, L: 0.2 μg/cm^2, Positive: sample treated with H_2O_2 300 nM, Negative: untreated cells.

Discussion

Epidemiological studies have established a direct correlation between the levels of ambient air particles and cardiopulmonary diseases resulting in an estimated 500,000 deaths each year worldwide (WHO, 1994). In recent study, there are many evidences that PM causes damage of DNA, protein and lipid [3,11]. However, there is no enough data about the comparative study between PM2.5 and PM10. The precise cellular and molecular mechanisms underlying the toxic pulmonary effects of PM are not fully established, either.

PM2.5 is known to have more heavy metals. In this reason, PM2.5 generates more metal-catalyzed reactive oxygen species [4,8,10,13] and PM2.5 influences severity of allergic airways disease [9]. Furthermore, PM2.5 is believed to cause more oxidative DNA damage than PM10, and PM2.5 may induce more damage to human health than PM10. Also, PM2.5 and PM10 demonstrated a different biological activity driven that PM2.5 was dominant by number and showed a greater abunance of C-rich particles.

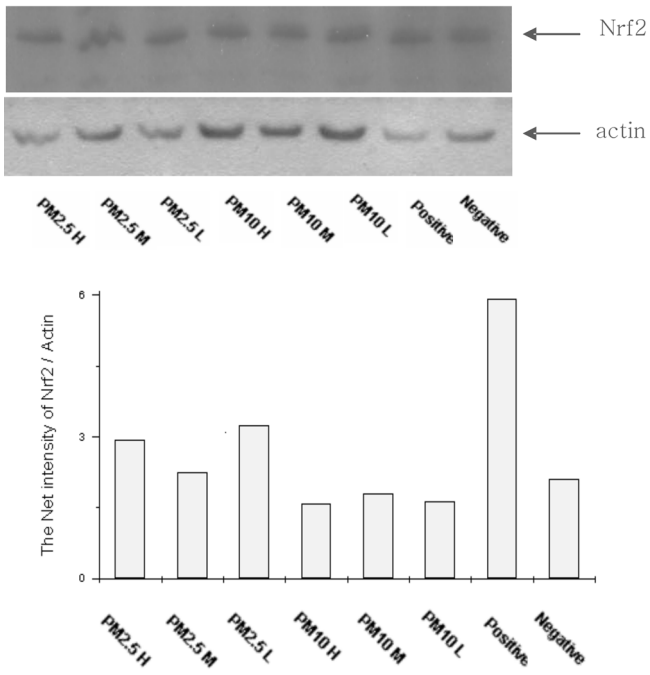

Fig. 12. Expression of Nrf2 protein in the PM2.5 - and PM10 - treated rat lung epithelial cell was determined by Western Blotting. The lower shows the ratio of Nrf2/actin using calculatging by densitometor. In the cell treated with PM2.5, the level of Nrf2 was much highly increased than in the cell treated PM10 (H: 5 μg/cm^2, M: 1 μg/cm^2, L: 0.2 μg/cm^2, Positive: 300 nM H_20_2, Negative: untreated cells).

PM2.5 also showed a greater surface area than PM10 at the same weight [18]. The purpose of this study is not only to compare *in vitro* toxicity of PM2.5 and PM10 collected in urban area of Seoul in rat lung epithelial cell, but also to provide the biological data for the risk assessment of PMs.

In MTT assay, PM2.5 has shown much highly cytotoxic than PM10 in rat lung epithelial cells and the similar results reported in alveolar epithelial cells [3]. The cytotoxicity could be related with apoptosis, probably due to different chemical components, especially heavy metals, between PM2.5 and PM10. In Dreher *et al.*' report [7] the percentage of heavy metals (As, Cr and Cd) was much higher in the constituents of fine particle (PM2.5) than coarse particle (PM10). Our data showed very similar results, suggesting that the different composition of heavy metals may be one of the underlying different toxicity in rat lung epithelial between PM2.5 and PM10.

Cellular level of MDA is a sensitive marker for oxidative damage, especially lipid peroxidation and has been widely used [24]. Recent study found that micro level of PM2.5 (not PM10) increased the MDA in human [23]. In this study, PM2.5 induced more MDA formation than PM10 suggesting that PM2.5 induced more oxidative stress than PM10. In the single strand breakage assay using pBR322 DNA, the ratio of changed form II/total plasmid DNA (form

I + form II) in PM2.5-treated DNA was significantly increased compared to both untreated-DNA and PM10-treated DNA ($P<0.05$). This is the evidence that PM2.5 induces more apoptotic change. Also, in this study, PM2.5 caused more apoptotic change than PM10. In the cell treated with PM2.5, DNA laddering patterns ware clearly found compared to PM10 treated cell. DNA laddering patterns indicate that apoptosis can be caused by PM2.5-induced ROS. These results suggest that PM2.5 induces more free radicals, which can induce DNA damage and apoptosis and this may be due to different ratio of heavy metals in PMs.

The genes investigated in this study include the genes for catalase, an important antioxidant enzyme that prevents the accumulation of intracellular hydrogen peroxide; Cu/Zn- and Mn-superoxide dismutase (Cu/Zn-SOD and Mn-SOD), the primary antioxidant enzymes that protect cells from oxidative damage by rapidly converting superoxide radicals into hydrogen peroxide, which is further detoxified by catalase and GPX; Mth1, DNA repair enzyme [15]. In the RT-PCR approach, the expression of catalase was decreased in the cell treated with PM2.5 in a concentration-dependent manner, suggesting the depletion of repair enzyme. Also, recent studies suggest that Mth1 gene expression may represent a molecular marker of oxidative stress that can be used to elucidate the temporal relationships between oxidative stress and the development of lung cancer [15].

Recent studies have shown that Nrf2 heterodimerizes with Jun (c-Jun, Jun-B, and Jun-D) proteins that bind with antioxidant response element (ARE) and regulate expression and induction of NADPH:quinone oxidoreductase and glutathione-S-transferase gene, encoding antioxidant enzyme genes [13]. Our data suggest that PM2.5 could cause more serious damage of DNA, cellular lipid, antioxidant enzyme than PM10 in rat lung epithelial cell. Therefore, PM2.5 might be more closely associated with PM-induced disease.

In conclusion, the PM2.5 may be more harmful to human than PM10 through oxidative stress. Therefore, the new standard have to focus on smaller particles that are likely responsible for adverse health effects.

Acknowledgment

This study was partly supported by BK21 project.

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