RESEARCH ARTICLE

Particulate matter 2.5 promotes inflammation and cellular dysfunction via reactive oxygen species/p38 MAPK pathway in primary rat corneal epithelial cells

Da Hye Kim\textsuperscript{a,b,*}, Hyesook Lee\textsuperscript{a,c}, Hyun Hwangbo\textsuperscript{a,d}, So Young Kim\textsuperscript{e,d}, Seon Yeong Jia\textsuperscript{d}, Min Yeong Kim\textsuperscript{a,d}, Seh-Kwang Park\textsuperscript{e,f}, Sung-Ho Park\textsuperscript{e,f}, Mi-Young Kim\textsuperscript{e,f}, Gi-Young Kim\textsuperscript{g}, Jaehun Cheong\textsuperscript{b}, Soo-Wan Nam\textsuperscript{h,i} and Yung Hyun Choi\textsuperscript{a,d,h,j}

\textsuperscript{a}Anti-Aging Research Center, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{b}Department of Molecular Biology, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{c}Department of Convergence Medicine, Pusan National University, Yangsan, Republic of Korea; \textsuperscript{d}Department of Biochemistry, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{e}Research and Development Department, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{f}BGN CARE Co Ltd., Seoul, Republic of Korea; \textsuperscript{g}Department of Marine Life Science, Jeju National University, Jeju, Republic of Korea; \textsuperscript{h}Department of Smart Bio-Health, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{i}Department of Biomedical Engineering and Biotechnology Major, Division of Applied Bioengineering, College of Engineering, Dong-Eui University, Busan, Republic of Korea; \textsuperscript{j}Core-Facility Center for Tissue Regeneration, Dong-Eui University, Busan, Republic of Korea

ABSTRACT

Purpose: Numerous studies have linked particulate matter 2.5 (PM\textsubscript{2.5}) to ocular surface diseases, but few studies have been conducted on the biological effect of PM\textsubscript{2.5} on the cornea. The objective of this study was to evaluate the harmful effect of PM\textsubscript{2.5} on primary rat corneal epithelial cells (RCECs) \textit{in vitro} and identify the toxic mechanism involved.

Materials and methods: Primary cultured RCECs were characterized by pan-cytokeratin (CK) staining. In PM\textsubscript{2.5}-exposed RCECs, cell viability, microarray gene expression, inflammatory cytokine levels, mitochondrial damage, DNA double-strand break, and signalling pathway were investigated.

Results: Exposure to PM\textsubscript{2.5} induced cytotoxicity and morphological changes in RCECs. In addition, PM\textsubscript{2.5} markedly up-regulated pro-inflammatory mediators but down-regulated the wound healing-related transforming growth factor-\(\beta\). Furthermore, PM\textsubscript{2.5} promoted mitochondrial reactive oxygen species (ROS) production and mediated cellular damage to mitochondria and DNA, whereas these cellular alterations induced by PM\textsubscript{2.5} were markedly suppressed by a potential ROS scavenger. Noteworthy, removal of ROS selectively down-regulated the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and the activation of the nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) p65 in PM\textsubscript{2.5}-stimulated cells. Additionally, SB203580, a p38 MAPK inhibitor, markedly suppressed these PM\textsubscript{2.5}-mediated cellular dysfunctions.

Conclusions: Taken together, our findings show that PM\textsubscript{2.5} can promote the ROS/p38 MAPK/NF-\(\kappa\)B signalling pathway and lead to mitochondrial damage and DNA double-strand break, which is ultimately caused inflammation and cytotoxicity in RCECs. These findings indicate that the ROS/p38 MAPK/NF-\(\kappa\)B signalling pathway is one mechanism involved in PM\textsubscript{2.5}-induced ocular surface disorders.

Introduction

Dust refers to particulate matter (PM) that floats in the atmosphere. PM is classified as PM\textsubscript{10} with size less than 10\,\mu m and PM\textsubscript{2.5} with size less than 2.5\,\mu m. Normal dust is not a big problem because it is filtered and discharged from the mucous membrane of the nose and the bronchus\textsuperscript{1}. However, fine dust is very small and cannot be filtered out, consequently it can be absorbed into the cells and tissues body \textit{via} breathing and skin\textsuperscript{1,2}. The concentration of fine dust is increasing day by day, causing serious air pollution\textsuperscript{3}. In January 2020, the average PM\textsubscript{2.5} exposure per population in Korea was 25\,\mu g/m\textsuperscript{3}, which was the highest among Organization for Economic Cooperation and Development (OECD) countries, and the severity of air pollution due to fine dust in Korea is gradually increasing\textsuperscript{3}. In this regard, the World Health Organization has been providing guidance on the concentration of fine dust in the air since 1987\textsuperscript{4}, and the International Agency for Research on Cancer designated fine dust as a Class 1 carcinogen in 2013\textsuperscript{5}. Accumulated evidences support that fine dust has toxic to biological organisms and that it is involved in the development of various diseases, including cancer, respiratory disease, neurological disorder, skin disease, and cardiovascular disease\textsuperscript{2,6-8}. Despite active research into the pathological mechanisms and toxicity caused by fine dust, studies on biological effects of fine dust on eyes and underlying damage mechanisms remain insufficient.

CONTACT Yung Hyun Choi choiyh@deu.ac.kr Department of Biochemistry, Dong-Eui University, 52-57, Yangjeong-ro, Busanjin-gu, Busan 47227, Republic of Korea

\*These authors contributed equally to this work as first author.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/15569527.2022.2122489.
Eye is naturally and directly exposed to the outside, especially to air pollutants including fine dust without any protection\textsuperscript{9}. Corneal disease is a major cause of blindness, and the surface of the eye is damaged by various factors\textsuperscript{10}. Trauma or infection by foreign objects may induce itchiness and pain of the eye, which may cause various ocular surface disorders, such as allergic keratitis, conjunctivitis, and dry eye syndrome\textsuperscript{11}. Accumulated epidemiological studies have shown that people often experience symptoms, such as itching, irritation, and foreign body when they are exposed to severe air pollution for a short term or a long term\textsuperscript{9}. In this respect, several studies have reported that air pollution is closely associated with an increase in the number of ophthalmic outpatients with allergic keratitis and conjunctivitis\textsuperscript{12-15}. Torricelli et al.\textsuperscript{16} have suggested that exposure to air pollution can break tear film stability and influence tear film osmolarity. Furthermore, decreased tear break time has been observed in subjects who are exposed to PM\textsubscript{2.5}, suggesting that PM\textsubscript{2.5} can result in instability of the tear layer and suppression of tear volume\textsuperscript{17}. Recently, a few studies have suggested the pathological mechanism of PM\textsubscript{2.5}-mediated ocular surface damage. Tan et al.\textsuperscript{18} have demonstrated that mice show characteristics of dry eye syndrome including detachment of corneal epithelium, destroy of the tear film, and inflammation of lacrimal gland after they are exposed to PM\textsubscript{2.5}. PM\textsubscript{2.5} can induce cytotoxic effects in corneal epithelial cells due to DNA damage, senescence\textsuperscript{19} and autophagic cell death caused by increased reactive oxygen species (ROS)\textsuperscript{14}. Large amounts of ROS can promote cellular damages and dysfunctions, such as DNA damage, mitochondrial damage, cell death, enzyme inactivation, and amino acid acidification\textsuperscript{20}. It is well-known that the generation of ROS by PM\textsubscript{2.5} exposure can cause a variety of cellular lesions\textsuperscript{21,22}. In CECs, house dust which contains fine dust can cause inflammation and mitochondrial DNA damages through ROS-mediated cytotoxicity\textsuperscript{23}. Furthermore, numerous studies demonstrated that PM triggers inflammation via ROS/mitogen-activated protein kinase (MAPK)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\kappa B) signalling pathway in bronchial epithelial cells\textsuperscript{24,25}. More recently, it has been established that PM\textsubscript{2.5} can promote delayed wound healing via interruption of focal adhesion kinase (FAK) signalling pathway, causing dry eye syndrome in C57BL/6 mice\textsuperscript{26}. Although there is a growing interest in the effect of exposure to fine dust on eyes with a few lab-scale experiments and epidemiological studies reporting toxicological effects of PM\textsubscript{2.5}, the underlying pathological mechanism remains unclear and more research studies are needed. Therefore, the aim of this study was to evaluate the biological effects of PM\textsubscript{2.5} on primary rat corneal epithelial cells (RCECs), and underlying mechanisms involved in the pathological effects were also explored.

**Materials and methods**

**PM\textsubscript{2.5} preparation**

Standard diesel PM\textsubscript{2.5} (SRM 1650b) was obtained from the National Institute of Standards and Technology (Gaithersburg, MD). The 1650b diesel PM2.5 was predominantly composed of heterocyclic polycyclic aromatic hydrocarbons (PAHs) and nitro-PAHs. As described previously\textsuperscript{9}, PM\textsubscript{2.5} was dissolved in dimethysulfoxide (DMSO; Invitrogen-Gibco, Carlsbad, CA) to prepare a 25 mg/mL stock solution, which was diluted in culture medium or normal saline just before use.

**Culture of primary rat corneal epithelial cells**

Female Sprague–Dawley rats (six weeks old) were obtained from Samtako Bio Korea (Osan, Republic of Korea) and acclimated for one week. This study was approved by the Institutional Animal Care and Use Committee of Dong-eui University (approval No. R2019-005). All procedures were followed in accordance with the guide for the Use of Animals in Ophthalmic and Vision Research. RCECs were isolated from specimens collected after surgical excision using a modified procedure\textsuperscript{27-29}. In brief, corneal buttons were cut from the eye and cleaned of extraneous tissues. They were then plated flat on a six-well plate with epithelium side up. After 10 min to allow for attachment of the explant, Mg\textsuperscript{2+} and Ca\textsuperscript{2+}-free Hank’s balanced salt solution (Thermo Fisher Scientific, Waltham, MA) containing 1.2 U dispase\textsuperscript{30} II (Sigma–Aldrich Chemical Co., St. Louis, MO) was added to each well and incubated at 37 °C for 10 min. Corneal epithelium was separated from buttons under a phase-contrast microscope (Carl Zeiss, Oberkochen, Germany) and incubated in keratocyte serum-free medium (KSF; Invitrogen-Gibco) containing 25 mg bovine pituitary extract and 2.5 μg human recombinant epidermal growth factor at 37 °C with 95% humidity and 5% CO\textsubscript{2}. The medium was changed every 2 d. After approximately 10 d, the explant was carefully transferred to a new dish. RCECs were subcultured using TrypLE Express (Thermo Fisher Scientific) at a split ratio of 1:3 after small cells reached subconfluence. Passaged cells were cultured in complex medium (1:1 ratio) with KSF and Dulbecco’s Modified Eagle’s medium/F12 (Thermo Fisher Scientific) medium supplemented with 10% foetal bovine serum (Thermo Fisher Scientific). Cells between 5th and 10th passages were used for all experiments. Cell morphology was observed under a phase-contrast microscope.

**CCK-8 assay**

To evaluate the cytotoxicity of PM\textsubscript{2.5} in RCECs, CCK-8 assay (Abcam Inc., Cambridge, UK) was performed according to the manufacturer’s instructions as previously described\textsuperscript{10}. Optical density values at 460 nm were measured with a microplate spectrophotometer (VERSA Max, Molecular Device Co., Sunnyvale, CA).

**NanoString nCounter® miRNA assay**

Microarray gene expression was performed using an nCounter Sprint platform (NanoString Technologies, Inc. Seattle, WA) as described previously\textsuperscript{31}. Briefly, RCECs were treated with 200 μg/mL PM\textsubscript{2.5} for 24 h and harvested. Total RNA was isolated from these cells using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer’s
instructions. RNA was then subjected to quality evaluation and quantitative analysis using an AATI fragment analyser (Agilent Technologies, Santa Clara, CA) and a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE). After solution-phase hybridization between the target mRNA and reporter-capture probe pairs, excess probe was removed. Probe/target complexes were aligned and immobilized in an nCounter cartridge (NCT-120), which was then placed in a digital analyser for image acquisition and data processing. After digital analysis, the raw data were normalized against housekeeping genes. Each gene expression change was expressed as a log-2 fold change value compared to control cells. A heat-map was generated to show the difference in gene expression that as red represents up-regulated genes and green represents down-regulated genes, respectively.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA kits for tumour necrosis factor-α (TNF-α; catalogue No. SMTA00B), interleukin-1β (IL-1β; catalogue No. MLB00C), and transforming growth factor-β (TGF-β; catalogue No. DB100B) were obtained from R&D system (Minneapolis, MN). Prostaglandin E2 (PGE2) assay kit (catalogue No. MLB00C), and 100 nM PM2.5 for 24 h. Immunofluorescence staining using pan-cytokeratin (CK) antibodies (catalogue No. DB100B) was purchased from Cayman Chemical (Ann Arbor, MI). Cytokines levels in culture supernatants were determined using these kits according to each manufacturer's instructions.

**Fluorescence staining**

To investigate intracellular ROS levels, cells were stained with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA; Thermo Fisher Scientific) dye as previously described. In order to investigate mitochondrial ROS levels, cells were stained with 5 μM MitoSOX™ red mitochondrial superoxide indicator (Thermo Fisher Scientific) dye images were then acquired with an EVOS Cell Imaging System (Thermo Fisher Scientific). To evaluate live mitochondrial mass, 100 nM MitoTracker® Red probe (Thermo Fisher Scientific) was used to stain cells and 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich Chemical Co.) was used to counterstain nuclei. After 30 min of staining, cells were fixed with 4% formaldehyde and observed using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) at the Core-Facility Centre for Tissue Regeneration, Dong-eui University (Busan, Republic of Korea).

**Immunofluorescence analysis**

To characterize primary RCECs, cells were subjected to immunofluorescence staining using pan-cytokeratin (CK) antibody. In another experiment, cells were treated with or without 1 mM N-acetyl-L-cysteine (NAC; Sigma-Aldrich Chemical Co.) at room temperature for 1 h. They were then treated with 100 μg/mL PM2.5 for 24 h. Immunofluorescence staining for phosphorylated histone H2AX (γH2AX) and phospho-NF-κB p65 was carried out as described previously. DAPI was used to counterstain the nuclei. Mounted cells were visualized using a fluorescence microscope. Antibodies used for this experiment are provided in Supplementary Table S1.

**Immunoblotting**

Cells were treated with or without 1 mM NAC and 10 μM SB203580 for 1 h, and then treated with 100 μg/mL PM2.5 for 24 h. Total protein was extracted using Pro-prep protein extraction solution (Intron Biotechnology, Seongnam, Republic of Korea). Nuclear and cytoplasmic proteins were isolated using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Fisher Scientific). As previously described, equal proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to Immun-Blot® PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Subsequently, these membranes were probed with specific primary anti-bodies (Supplementary Table S1). The membranes were then exposed enhanced chemiluminescence solution (Thermo Fisher Scientific) and visualized using a Fusion FX Imaging System (Vilber Lourmat, Torcy, France). Densitometric analysis of the bands was performed using the ImageJ® software version 1.48 (NIH, Bethesda, MD).

**Statistical analysis**

All experiments were performed at least three times. Data are expressed as mean ± standard deviation (SD). GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA) was used for all statistical analyses. Significant differences were analysed using analysis of variance (ANOVA) followed by Tukey’s test. Probability values of p < 0.05 were considered as statistically significant.

**Results**

**Characterization of RCECs**

Some fibroblasts were observed in early-passage (<passage 5) RCECs (Figure 1(A)). There was a heterogenous population of small and large squamous cells. Partially, large cells made up blebs near the tissue were detached from the culture surface. Meanwhile, between the 5th passage and the 10th passage, uniformly small cells with topical cobblestone morphology similar to stable primary corneal epithelial cell lines obtained from human and murine were observed. However, cells with passage number more than 15 underwent senescence and showed morphological changes. Therefore, we used stable cells with passage number between 5 and 10 for all experiments. To determine whether cultured RCECs have properties of epithelial cells derived from cornea, cells were stained with pan-CK antibody. Figure 1(B) showed that RCECs strongly expressed pan-CK, a positive marker for corneal epithelial cells. These results confirmed that RCECs used in this study were derived from the cornea.
PM2.5 promotes cytotoxicity in RCECs

Cytotoxicity of PM2.5 to primary RCECs was evaluated using CCK-8 assay. As shown in Figure 1(C), PM2.5 had no cytotoxicity to primary RCECs at concentration up to 50 g/mL. However, viabilities of cells treated with PM2.5 at concentration above 100 g/mL were significantly decreased compared to those of normal cells. After treatment with PM2.5 at 100 and 200 g/mL, viabilities of RCECs were approximately 83 and 50%, respectively. In addition, PM2.5 caused morphological changes including cell membrane collapse and aggregation (Figure 1(D)). These results suggest that PM2.5 can induce morphological changes of RCECs, and it is cytotoxic to RCECs at high concentrations.

PM2.5 increases the expression of inflammation-related mediators

To evaluate the effect of PM2.5 on gene expression, NanoString nCounter® miRNA gene analysis was performed. Results of gene microarray analysis of RCECs exposed to 200 g/mL PM2.5 compared to normal cells are shown as heatmaps. Expression of each gene was converted to a log2-fold-change value. If the value showed an increase of more than 3-fold, it was marked in red. If it showed a decrease of more than 3-fold, it was marked in green (Figure 2(A)). Figure 2(B) shows results of quantification based on the log2-fold change values of gene expression. Overall, PM2.5-treated cells greatly enhanced the expression of genes involved in the inflammatory response. In addition, changes in the expression of chemokine receptors and chemokine ligands were observed in PM2.5-treated cells. The expression of Toll-like receptor (TLR) gene involved in innate immunity was upregulated by PM2.5, and the expression of its corresponding immune-related gene was also increased by PM2.5. Furthermore, expression levels of genes involved in inflammatory responses, including those in MAPK and nuclear translocation factor NF-κB signalling pathways, were enhanced by PM2.5. However, the expression of TGF-β gene known to contribute to wound healing and repair response was markedly down-regulated in PM2.5-treated cells. Based on the altered mRNA expression of the inflammatory factor induced by PM2.5, we verified that secretion levels of major inflammatory cytokines including TNF-α, IL-1β, and PGE2 were upregulated by PM2.5. PM2.5 treatment increased cytokine production in RCECs compared to the control without such treatment. Figure 2(C) shows that TNF-α, IL-1β, and PGE2 secretion levels were increased by 1.3-fold, 1.75-fold, and 1.36-fold, respectively, following treatment with 200 g/mL of PM2.5 compared to those in control cells. Results of nCounter mRNA analysis revealed that the secretion level of TGFβ was significantly decreased to 0.4-fold of control in 200 g/mL PM2.5-treated cells. These results suggest that PM2.5 can markedly up-regulate the expression and secretion of pro-inflammatory mediators while down-regulating the mRNA expression and secretion of wound healing-related TGFβ in RCECs.

PM2.5 induces intracellular ROS production and cellular organelle damages

To investigate whether PM2.5 might affect intracellular ROS production, RCECs were stained with DCF-DA and MitoSOX®
Figure 2. PM$_{2.5}$ increases the expression and secretion of inflammatory mediators in RCECs. (A and B) Heatmap of candidate gene expression using NanoString nCounter miRNA expression assay in PM$_{2.5}$-stimulated RCECs. Cells were treated with or without 200 µg/mL of PM$_{2.5}$ for 24 h. Total RNA was isolated and hybridization was performed using a reporter probe and a capture probe. (A) Heatmap representing expressed genes with fold-change cut-off of 3.0 (upregulation and downregulation in red and green, respectively). (B) Expression of each gene was indicated as fold change compared to the control. (C) Levels of cytokines including TNF-α, IL-1β, PGE$_2$, and TGF-β1 in supernatants of PM$_{2.5}$-stimulated RCECs. Three independent experiments were performed in duplicate. The data are expressed as the means ± SD (n = 6). *p < 0.05; **p < 0.01; and ***p < 0.001 between groups. PM$_{2.5}$: particulate matter 2.5; RCECs: rat corneal epithelial cells; TNF-α: tumour necrosis factor; IL-1β: interleukin-1β; PGE$_2$: prostaglandin E2; TGF-β1: transforming growth factor-β1.

red. Figure 3(A) shows that intracellular and mitochondrial ROS levels are markedly enhanced by PM$_{2.5}$ dose-dependently. Next, we evaluated the effect of PM$_{2.5}$ on mitochondria and nuclei of RCECs. Many MitoTracker Red-positive cells indicating live mitochondria were observed in control cells. However, these cells were substantially decreased in PM$_{2.5}$-treated group (Figure 3(B)). In addition, the expression of γH2AX, a marker of DNA double-strand break, was notably increased by PM$_{2.5}$ (Figure 3(C)). These results indicate that PM$_{2.5}$ can promote intracellular ROS production and mediate cellular damage by affecting mitochondria and DNA double-strand break in RCECs.
PM2.5 activates mitogen-activated protein kinase (MAPK) and NF-κB signalling pathway

To explore signalling pathways involved in PM2.5-induced inflammation and cellular damages, Western blot analysis, and immunofluorescence staining were performed. Figure 4(A,B) shows that the phosphorylation of p38 MAPK markedly up-regulated by PM2.5, and the levels sustained from 1 to 24 h after exposur. As shown in Figure 4(C,D), PM2.5 up-regulated the expression of extracellular signal-regulated kinase (ERK) and the phosphorylation of p38 MAPK, but slightly down-regulated the expression of c-Jun N-terminal kinase (JNK). Meanwhile, the expression and phosphorylation of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (AKT) did not alter after treatment with PM2.5. Furthermore, we determined whether NF-κB signalling pathway was activated by PM2.5 in RCECs. The result of Western blot analysis showed that the expression of NF-κB p65 in the nucleus fraction was markedly increased by PM2.5 in a time and a dose-dependent manner (Figure 4(E–H)). This result is consistent with the result of immunofluorescence staining suggesting that the phosphorylated form (p)-NF-κB p65 was strikingly expressed in nucleus (Figure 4(I)). These results suggest that PM2.5 can activate MAPK signalling pathway and markedly induce the translocation of NF-κB p65 into the nucleus from the cytoplasm.

PM2.5-induced cellular damage is triggered by ROS in RCECs

Based on the above findings that PM2.5 induced intracellular ROS production, we next evaluated the role of ROS in PM2.5-mediated cellular dysfunction including cytotoxicity, inflammation, and organelle damages. To determine the effect of blocking ROS in PM2.5-stimulated RCECs, cells were pre-treated with NAC, a potential ROS scavenger, before exposure to PM2.5. As a result of CCK-8 assay, NAC significantly recovered PM2.5-induced decrement of cell viability (Figure 5(A)). Furthermore, pre-treatment with NAC prominently decreased morphological changes such as cell membrane collapse following PM2.5 (Figure 5(B)). In addition, PM2.5-mediated increases of TNF-α, IL-1β, and PGE2 levels were significantly suppressed by NAC treatment, leading to similar to those in control cells (Figure 5(C)). Moreover, removal of ROS by NAC remarkably decreased the population of DCF-DA-positive cells and MitoTracker® Red-positive cells (Figure 5(D), top and middle panels). Additionally, NAC completely suppressed the expression of p-NF-κB p65 in the nucleus compared to PM2.5-treated cells (Figure 5(D), bottom panels). Noteworthy, blocking ROS with NAC markedly down-regulated the phosphorylation of p38 MAPK without affecting the expression or phosphorylation of ERK and JNK (Figure 5(E,F)). As shown in Figure 5(G), NAC also repressed the expression of p-NF-κB p65 in the nucleus compared to PM2.5-treated cells. These results suggest that removal of ROS by NAC can markedly suppress PM2.5-mediated cellular damages including cytotoxicity, inflammation, mitochondrial damage, and DNA double-strand break. More importantly, ROS blocking selectively down-regulated p38 MAPK signal and inactivated NF-κB p65 in PM2.5-stimulated cells.

PM2.5-induced cellular dysfunction involves ROS/p38 MAPK signalling pathway in RCECs

Since NAC specifically suppressed the phosphorylation of p38 MAPK in PM2.5-stimulated RCECs, we next investigate whether p38 MAPK was directly involved in PM2.5-mediated cellular dysfunction. As shown in Figure 6(A), SB203580, a selective inhibitor of p38 MAPK, significantly suppressed

---

**Figure 3.** PM2.5 induces mitochondrial ROS generation and cellular organelle damages in RCECs. (A) RCECs were treated with indicated concentrations of PM2.5 for 30 min and co-stained with 10 μM DCF-DA and 5 μM MitoSOX™ Red dye. ROS production was observed under a fluorescence microscope. Scale bar: 75 μm. (B) RCECs were treated with PM2.5 for 24 h and then stained with 100 nM MitoTracker® Red dye (red) for 10 min. Scale bar: 75 μm. (C) RCECs were treated with indicated concentration of PM2.5 for 24 h and then subjected to immunofluorescence staining with anti-γH2AX (Ser139) antibody (red). Scale bar: 200 μm. (B and C) Nucleus was counterstained with DAPI (blue). All experiments were performed three independent times. PM2.5: particulate matter 2.5; ROS: reactive oxygen species; RCECs: rat corneal epithelial cells; DCF-DA: dichlorodihydrofluorescein diacetate; γH2AX: phosphorylated histone H2AX; DAPI: 4',6'-diamidino-2-phenylindole.
PM2.5-induced cytotoxicity. Furthermore, pre-treatment with SB203580 remarkably decreased mitochondrial damage and levels of pro-inflammatory cytokines following exposure to PM2.5 (Figure 6(B,C)). Additionally, p38 MAPK inhibition greatly blocked the expression of p-NF-κB p65 in the nucleus (Figure 6(D)). These results suggest that p38 MAPK signalling pathway is activated after ROS generation in RCECs following PM2.5 treatment.

**Discussion**

The front of the eye is protected by tear film that covers the entire eye.\(^{39}\) Accumulating evidences have shown that PM can aggravate dry eye disease or allergic keratitis and conjunctivitis, causing ocular discomfort and inflammation.\(^{7,40,41}\) Dry eye syndrome is a representative ocular surface disease caused by a variety of factors including air pollution, changes in visual acuity, and instability of tear film. It is often accompanied by potential ocular surface damage, tear osmotic pressure elevation, and ocular inflammation.\(^{40}\) Damage to the ocular surface epithelium by various factors including fine dust can trigger an inflammatory response that may lead to further decrease of tear production and worsening of symptoms.\(^{42,43}\) Herein, we verified that results of this study revealed that RCECs could recognize PM2.5 as an external harmful substance, thus generating immune responses. PM2.5 up-regulated the expression of genes involved in inflammation and immune response. Among pro-inflammatory cytokines, TNF-\(\alpha\), IL-1\(\beta\), and PGE\(_2\) showed significantly increased secretion levels in RCECs after treatment with PM2.5. However, PM2.5 markedly decreased the expression and secretion level of TGF-\(\beta\) known to play a critical role in wound healing.\(^{44}\) Several studies have also reported that PM2.5 can induce inflammatory responses in corneal epithelial cells.\(^{18,26,29}\) Ma et al.\(^{29}\) have reported that PM2.5 treatment significantly elevated mRNA and protein levels of interferon...
gamma (IFN-γ), IL-10, IL-17, and IL-21 in human bronchial epithelial cells. They have suggested that increases of cytokines require the presence of macrophages and that PM2.5 can promote cytotoxic inflammation of T cells in a macrophage-dependent manner. Another study has shown that topical exposure to PM2.5 increased the expression levels of pro-inflammatory cytokines, including TNF-α and IL-6, in murine model. In addition, PM2 can increase oxidative stress and inflammation through the NF-κB/TNF-α pathway. NF-κB can increase transcriptional activities of various pro-inflammatory genes including cytokines and chemokines, thus playing a key role in inflammatory regulation. Numerous studies have shown that the expression of NF-κB p65 protein is increased in corneal tissues of PM2.5 and PM10 injected mice showing clinical symptoms similar to dry eye syndrome. In this study, PM2.5 caused inflammatory responses with NF-κB strongly expressed in the nucleus of PM2.5-treated cells. In addition, Western blot analysis revealed that p-NF-κB p65

Figure 5. PM2.5-induced cellular damages triggered by ROS in RCECs. Cells were pre-treated with 1 mM NAC for 1 h before stimulation with 100 μg/mL of PM2.5 for 24 h. (A) Effect of NAC on viability of PM2.5-stimulated cells. (B) Representative morphological images acquired with an inverted microscope. Scale bar: 20 μm. (C) Effect of NAC on PM2.5-stimulated levels of pro-inflammatory cytokines. (A and C) Three independent experiments were performed in triplicate. The data are expressed as the means ± SD (n = 9). *p < 0.05; **p < 0.01; and ***p < 0.001 compared to control. #p < 0.05; ##p < 0.01; and ###p < 0.001 compared to PM2.5-treated cells. (D, top panels) Effect of NAC on PM2.5-induced mitochondrial ROS production. Scale bar: 75 μm. (D, middle panels) Effect of NAC on mitochondria in PM2.5-stimulated cells. Merged images of MitoTracker® Red dye (red) and DAPI (blue) are shown. Scale bar: 75 μm. (D, bottom panels) Effect of NAC on PM2.5-induced DNA damage. Merged images of γH2AX (red) and DAPI (blue) are shown. Scale bar: 25 μm. (E) Effect of NAC on PM2.5-mediated alteration of MAPK signalling pathway. Expression and phosphorylation of MAPK signalling molecules are shown. (F) Bar graphs indicate the relative band density of the phosphorylated protein/non phosphorylated protein ratio in western blot analysis. Significant differences compared to the control cells (***p < 0.01) or PM2.5-treated cells (##p < 0.01). (G) Effect of NAC on PM2.5-induced NF-κB activation. Immunofluorescence images for p-NF-κB p65 (red) and DAPI (blue) are shown. Scale bar: 75 μm. All experiments were performed three independent times. PM2.5: particulate matter 2.5; ROS: reactive oxygen species; RCECs: rat corneal epithelial cells; NAC: N-acetyl-L-cysteine; DAPI: 4',6'-diamidino-2-phenylindole; γH2AX: phosphorylated histone H2AX; MAPK: mitogen-activated protein kinase; NF-κB: nuclear factor-κB; p-NF-κB: phosphorylated nuclear factor-κB.
was translocated into the nucleus from the cytoplasm. This result suggests that PM$_{2.5}$ can activate NF-κB-mediated inflammatory responses in RCECs.

PM$_{2.5}$ contains heterocyclic PAH, nitro-PAH, and various metals (iron, copper, chromium, vanadium, etc.) that can catalyse the Fenton reaction to generate ROS belonging to an active oxygen group. Actually, SRPM1650b was predominantly composed of heterocyclic PAHs and nito-PAHs. ROS are highly reactive molecules due to the chemistry of oxygen which does not share an electron pair. In human skin keratinocytes, PM$_{2.5}$ can activate NF-κB by intracellular ROS induction through interaction with TLR5 and nicotinamide adenine dinucleotide phosphate oxidase. In 2016, Xiang et al. suggested that ROS removal might be effective in protecting corneal epithelial cells from damage by PM$_{2.5}$. In this regard, our current findings also showed that removal of
ROS by NAC markedly suppressed PM2.5-mediated cellular damages including cytotoxicity, inflammation, mitochondrial damage, and DNA double-strand break. These findings demonstrate role of ROS in PM2.5-mediated cellular damages in RCECs. Specifically, ROS blocking selectively down-regulated p38 MAPK signal and caused in inactivation of NF-κB p65 in PM2.5-stimulated cells. The cascade of MAPKs signalling pathways is involved in a variety of cellular functions including cell proliferation, differentiation, migration, and survival. Among various MAPKs, p38 MAPK is strongly activated by environmental stresses, inflammatory cytokines, and various extracellular stimuli. There has been reported that PM can lead to injury in human lung endothelial cells via the ROS/p38 MAPK-dependent pathway and that the disruption to the endothelium can be attenuated by NAC. Another study has also reported that SB203580, a selective inhibitor of p38 MAPK, can inhibit the proliferation of vascular smooth muscle in PM2.5-mediated atherosclerosis model, indicating that the p38 MAPK signalling pathway plays a critical role in PM2.5-induced pathogenesis. More recently, it has been suggested that PM2.5-induced inflammation can activate the ROS/p38 MAPK pathway in human skin keratinocytes. Lee et al. have demonstrated that the ROS/p38 MAPK/protein kinase B pathway is involved in PM2.5-induced inflammatory lung injury and vascular hyperpermeability. Thus, PM2.5 is expected to activate the p38 MAPK signalling pathway with overall effects on various cells and organs. In this study, we found that the p38 MAPK signalling pathway was activated as a down-stream event of ROS under PM2.5-stimulated condition in RCECs. Furthermore, blocking the p38 MAPK signalling pathway by SB203580 significantly suppressed PM2.5-induced cytotoxicity, mitochondrial damage, and levels of pro-inflammatory cytokines. Therefore, exposure to PM2.5 can lead to the production of ROS which then activates the p38 MAPK signalling pathway, causing cellular dysfunction in RCECs.

Overall, this study suggests that PM2.5 can increase intracellular ROS to trigger the activation of the p38 MAPK signalling pathway, ultimately resulting in inflammation through NF-κB translocation into the nucleus. Furthermore, PM2.5 could activate the ROS/p38 MAPK signalling pathway and lead to mitochondrial damage and DNA double-strand break, causing cytotoxicity (Figure 7). Taken together, these findings suggest that the ROS p38 MAPK/NF-κB signalling pathway is one of the mechanisms involved in PM2.5-induced ocular surface disorders.

Geolocation information
The research was conducted in Busan, Republic of Korea.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This research was funded by the Basic Science Research Program through the National Research Foundation [2019R1C1C1008623 and 2021R1A2C200954911] and BGN Eye Clinic.
References

1. Baroody FM. How nasal function influences the eyes, ears, sinuses, and lungs. Proc Am Thorac Soc 2011;8:53–61.
2. Ritz B, Hoffmann B, Peters A. The effects of fine dust, ozone, and nitrogen dioxide on health. Dtsch Arztebl Int 2019;51:52–881–886.
3. OECD. Environment at a glance. Air quality. 2020. Available from: http://www.oecd.org/environment/env-at-a-glance.
4. Han L, Zhou W, Pickett ST, et al. Multicontaminant air pollution in Chinese cities. Bull World Health Organ 2018;96:233–242E.
5. Loomis D, Huang W, Chen G. The International Agency for Research on Cancer (IARC) evaluation of the carcinogenicity of outdoor air pollution: focus on China. Chin J Cancer 2014;33:189–196.
6. Santibañez-Andrade M, Chirino YL, González-Ramírez I, et al. Deciphering the code between air pollution and disease: the effect of particulate matter on cancer hallmarks. Int J Mol Sci 2019;21:136.
7. Kim H, Kim WH, Kim YY, et al. Air pollution and central nervous system disease: a review of the impact of fine particulate matter on neurological disorders. Front Public Health 2020;8:575330.
8. Lee H, Kim DH, Kim JH, et al. Urban aerosol particulate matter promotes necrosis and autophagy via reactive oxygen species-mediated cellular disorders that are accompanied by cell cycle arrest in retinal pigment epithelial cells. Antioxidants 2021;10:149.
9. Jung SJ, Mehta JS, Tong L. Effects of environment pollution on the ocular surface. Ocul Surf 2018;6:198–205.
10. Barrientes B, Nicholas SE, Wheelchel A, et al. Corneal injury: clinical and molecular aspects. Exp Eye Res 2019;186:107709.
11. Klopfner J. Effects of environmental air pollution on the eye. J Am Optom Assoc 1989;60:773–778.
12. Hyun SW, Song SJ, Park B, et al. Toxicological effects of urban particulate matter on corneal and conjunctival epithelial cells. Toxicol Res 2020;36:311–318.
13. Fu Q, Mo Z, Lyu D, et al. Air pollution and outpatient visits for conjunctivitis: a case–crossover study in Hangzhou, China. Environ Pollut 2017;231:344–350.
14. Fu Q, Lyu D, Zhang L, et al. Airborne particulate matter (PM$_{2.5}$) triggers autophagy in human corneal epithelial cell line. Environ Pollut 2017;227:314–322.
15. Szszyzko M, Kousha T, Castner J. Air pollution and emergency department visits for conjunctivitis: a case–crossover study. Int J Occup Med Environ Health 2016;29:381–393.
16. Torricelli AA, Novaes P, Matsuda M, et al. Correlation between signs and symptoms of ocular surface dysfunction and tear osmolarity with ambient levels of air pollution in a large metropolitan area. Cornea 2013;32:e11–e15.
17. Mo Z, Fu Q, Lyu D, et al. Impacts of air pollution on dry eye disease among residents in Hangzhou, China: a case–crossover study. Environ Pollut 2019;246:183–189.
18. Tang G, Li J, Yang Q, et al. Air pollutant particulate matter 2.5 induces dry eye syndrome in mice. Sci Rep 2018;8:17828.
19. Gao ZX, Song XL, Li SS, et al. Assessment of DNA damage and cell senescence in corneal epithelial cells exposed to airborne particulate matter (PM$_{2.5}$) collected in Guangzhou, China. Invest Ophthalmol Vis Sci 2016;57:3093–3102.
20. Zorov DB, Plotnikov EF, Jankauskas SS, et al. The phenoptosis problem: what is causing the death of an organism? Lessons from acute kidney injury. Biochemistry (Mosc) 2012;77:742–753.
21. Lee W, Choo S, Sim H, et al. Inhibitory activities of ononin on particulate matter-induced oxidative stress. Biotechnol Bioprocess E 2021;26:208–215.
22. Lee H, Hwangbo H, Ji SY, et al. Diesel particulate matter 2.5 promotes epithelial-mesenchymal transition of human retinal pigment epithelial cells via generation of reactive oxygen species. Environ Pollut 2020;262:114301.
23. Xiang P, He RW, Han YH, et al. Mechanisms of house dust-induced toxicity in primary human corneal epithelial cells: oxidative stress, proinflammatory signaling and mitochondrial dysfunction. Environ Int 2016;89:9030–37.
24. Wang J, Huang J, Wang L, et al. Urban particulate matter triggers lung inflammation via the ROS-MAPK-NF-kB signaling pathway. J Thorac Dis 2017;9:4398–4412.
25. Zeng Y, Zhu G, Zhu M, et al. Edaravone attenuated particulate matter-induced lung inflammation by inhibiting ROS-NF-kB signaling pathway. Oxid Med Cell Longev 2022;2022:690884.
26. Cui YH, Hu XZ, Gao ZX, et al. Airborne particulate matter impairs corneal epithelial cell migration via disturbing FAK/ROA signaling pathway and cytoskeleton organization. Nanotoxicology 2148;124:312–324.
27. Forbes DJ, Pozos RS, Nelson JD, et al. Characterization of rat corneal epithelium maintained in tissue culture. Curr Eye Res 1984;3:1471–1479.
28. Kobayashi T, Yoshioka R, Shiraisi A, et al. New technique for culturing corneal epithelial cells of normal mice. Mol Vis 2009;15:1589–1593.
29. Ma QY, Huang DY, Zhang HJ, et al. Exposure to particulate matter 2.5 (PM$_{2.5}$) induced macrophage-dependent inflammation, characterized by increased Th1/Th17 cytokine secretion and cytotoxicity. Int Immunopharmacol 2017;50:139–145.
30. Gu J, Wang J, You A, et al. MiR-137 inhibits the proliferation, invasion and migration of glioma via targeting to regulate EZH2. Genes Genomics 2021;43:1157–1165.
31. Ji SY, Lee H, Hwangbo H, et al. A Novel peptide oligomer of bacitracin induces M1 macrophage polarization by facilitating Ca$^{2+}$ influx. Nutrients 2020;12:1603.
32. Chae BS. Effect of low-dose corticosteroid pretreatment on the production of inflammatory mediators in super-low-dose LPS-primed immune cells. Toxicol Res 2021;37:47–57.
33. Liang Y, Kong D, Zhang Y, et al. Fisetin inhibits cell proliferation and migration of glioma independent of ROS. Ijms 2021;22:1361.
34. Pan Q, Ge F, Grigoryan A, et al. Correlation of DNA double-strand break repair and DNA damage responses in human thyroid TPC-1 cancer cells. Biotechnol Bioproc E 2020;25:197–205.
35. Kim DH, Kim JH, Hwangbo H, et al. Spermidine attenuates oxidative stress-induced apoptosis via blocking Ca$^{2+}$ overload in retinal pigment epithelial cells independently of ROS. Ijms 2021;22:1361.
36. Choi MJ, Mukherjee S, Yun JW. Loss of ADAMTS15 promotes browning in 3T3-L1 white adipocytes via activation of J3-adrenergic receptor. Biotechnol Bioproc E 2021;26:188–200.
37. Ma X, Shimamura S, Miyashita H, et al. Long-term culture and growth kinetics of murine corneal epithelial cells expanded from single corneas. Invest Ophthalmol Vis Sci 2009;50:2716–2721.
38. Lee HS, Lee JH, Yang JW. Effect of porcine chondrocyte-derived extracellular matrix on the periphery in mouse model. Graefeas Arch Clin Exp Ophthalmol 2014;252:609–618.
39. Kinner A, Wu W, Staudt C, et al. Gamma-H2AX in recognition and signaling of DNA double-strand breaks in the context of chromatin. Nucleic Acids Res 2008;36:5678–5694.
40. Tutt R, Bradley A, Begley C, et al. Optical and visual impact of tear film osmolarity and mucin content on the ocular surface. Invest Ophthalmol Vis Sci 2000;41:1147–1123.
41. Idarraga MA, Guerrero JS, Mosle SG, et al. Relationships between short-term exposure to an indoor environment and dry eye (DE) symptoms. J Clin Med 2020;9:1316.

42. Lee H, Jeon S, Kim CE, et al. A new ophthalmic pharmaceutical formulation, topical sulglycotide, enhances the ocular mucin secretion in desiccation stress-mediated dry eye disease. Invest Ophthalmol Vis Sci 2019;60:1076–1087.

43. Pflugfelder SC, Stern ME. Mucosal environmental sensors in the pathogenesis of dry eye. Expert Rev Clin Immunol 2014;10:1137–1140.

44. Tandon A, Tovey JC, Sharma A, et al. Role of transforming growth factor Beta in corneal function, biology and pathology. Curr Mol Med 2010;10:565–578.

45. Zhu J, Zhao Y, Gao Y, et al. Effects of different components of PM2.5 on the expression levels of NF-κB family gene mRNA and inflammatory molecules in human macrophage. Int J Environ Res Public Health 2019;16:1408.

46. Baldwin AS. Jr. The NF-κB and IκB proteins: new discoveries and insights. Annu Rev Immunol 1996;14:649–683.

47. Park JH, Troxel AB, Harvey RG, et al. Polycyclic aromatic hydrocarbon (PAH) o-quinones produced by the aldo-keto-reductases (AKRs) generate abasic sites, oxidized pyrimidines, and 8-oxo-dGuo via reactive oxygen species. Chem Res Toxicol 2006;19:719–728.

48. Dröge W. Free radicals in the physiological control of cell function. Physiol Rev 2002;82:47–95.

49. Ryu YS, Kang KA, Piao MJ, et al. Particulate matter induces inflammatory cytokine production via activation of NFκB by TLR5-NOX4-ROS signaling in human skin keratinocyte and mouse skin. Redox Biol 2019;21:101080.

50. Geest CR, Coffer PJ. MAPK signaling pathways in the regulation of hematopoiesis. J Leukoc Biol 2009;86:237–250.

51. Zarubin T, Han J. Activation and signaling of the p38 MAP kinase pathway. Cell Res 2005;15:11–18.

52. Wang T, Chiang ET, Moreno-Vinasco L, et al. Particulate matter disrupts human lung endothelial barrier integrity via ROS- and p38 MAPK-dependent pathways. Am J Respir Cell Mol Biol 2010;42:442–449.

53. Wan Q, Liu Z, Yang Y. Puerarin inhibits vascular smooth muscle cells proliferation induced by fine particulate matter via suppressing of the p38 MAPK signaling pathway. BMC Complement Altern Med 2018;181:146.

54. Kim JH, Kim M, Kim JM, et al. Afzelin suppresses proinflammatory responses in particulate matter-exposed human keratinocytes. Int J Mol Med 2019;43:2516–2522.

55. Lee W, Ku SK, Kim JE, et al. Inhibitory effects of protopanaxatriol type ginsenoside fraction (Rg3x65) on particulate matter-induced pulmonary injury. J Toxicol Environ Health A 2019;825:338–350.