ORIGINAL ARTICLE

Toxicological effects of urban particulate matter on corneal and conjunctival epithelial cells

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Abstract

Exposure to urban particulate matter (UPM) is a high-risk factor for various ocular surface diseases, including dry eye syndrome. However, the effects of UPM on corneal and conjunctival epithelium damage have not been fully elucidated. In this study, we investigated the toxicological effects of UPM exposure at high concentrations by using in vitro cultures. The cell viability, mucin expression, and the secreted inflammatory mediators of corneal and conjunctival epithelial cells was observed at 24 h after exposure to UPM. The progression of cell cycle was also examined by flow cytometry at 24 h after exposure to UPM. UPM reduced cell viability in a dose-dependent manner and increased cell population in S and G2 phase. The expression of mucin-1 was attenuated by UPM exposure, but that of mucin-4 was not. UPM increased interleukin (IL)-6 release and decreased IL-8 release. The intensity of 2',7'-dichlorofluorescein diacetate (DCF-DA) was highest at 4 h of UPM exposure. In conclusion, these results suggest that UPM causes the disruption of corneal and conjunctival epithelium by decreasing cell viability, altering cell cycle, disrupting mucin, and regulating inflammatory mediators.

Keywords Cell cycle · Cell death · Corneal/conjunctival epithelial cells · Mucin · Urban particulate matter

Abbreviations

CCCK-8	Cell Counting Kit-8
DES	Dry eye syndrome
DEP	Diesel exhaust particles
ELISA	Enzyme-linked immunosorbent assay
IL	Interleukin
nitro-PAHs	Nitro-substituted polycyclic aromatic hydrocarbons
PAHs	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyl
ROS	Reactive oxygen species
UPM	Urban particulate matter
WHO	World Health Organization

Introduction

Dry eye syndrome (DES) is one of the most prevalent ocular surface diseases associated with quality of life [1]. Globally, more than 30% of the adult population suffer from DES at various ages [2]. Damage to the ocular surface including the cornea and conjunctiva is the most common cause of DES [2]. Air pollutants including gases (such as ozone and NO₂), particulate matter, and some indoor hazards has a critical role in damaging ocular surface, triggering immune response [3]. It has been reported that inflammatory cytokines and mucin production induced by environmental factors including diesel exhaust particles (DEP) in human corneal and conjunctival epithelial cells [4].

Particulate matter is the general term used to describe particles present in ambient atmospheric air. Globally, particulate matter is becoming a serious problem, which can cause or aggravate diseases of the skin, eyes, and so on. The modern age is an urbanized society, and many people live in cities. Thus, it is important to address potential risk-based exposure assessment to urban particulate matter (UPM) in human health. Recently, the particulate matter was classified as carcinogenic (Group 1) to humans by the International Agency for Research on Cancer, an inter-governmental agency forming part of the World Health

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Organization [5]. According to Aphekom project, as the long-term exposure to PM_{2.5} decreased by 10 µg/m³, 22 months of life expectancy were added up for European persons 30 years of age and older [6]. It is also reported that the exposure of PM_{2.5} contributed to an additional deaths (11,924 Korean persons) of annual deaths in 2015 [7].

UPM plays a role in autophagy in human bronchial epithelial cells promoting inflammatory and oxidative effects [8]. Several studies reported that UPM causes various respiratory diseases [9–11], but a relationship between UPM and eye disease including DES has not been fully elucidated. In addition, few studies have reported about the mechanism linking UPM and cornea/conjunctiva. In this study, therefore, we investigated the adverse effects of UPM on corneal and conjunctival epithelial cells and its mechanism.

Materials and methods

Materials

UPM (NIST 1648a) was purchased from Sigma-Aldrich (St Louis, MO, USA). UPM (NIST SRM 1648a) was obtained from an urban area in St. Louis, MO, USA over a period greater than 12 months using a baghouse specially designed for this purpose [12]. The mean particle diameter of UPM is about 5.85 µm, and UPM contains polycyclic aromatic hydrocarbons (PAHs), nitro-substituted PAHs (nitro-PAHs), polychlorinated biphenyl (PCB) congeners, and chlorinated pesticides.

Cell culture

Human conjunctival epithelial cells (clone 1-5c-4, CCL-20.2™) and human corneal epithelial cells [HCE-2 (50.B1), CRL-11135™] were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). The conjunctival epithelial cells were maintained at 37 °C in RPMI 1640 containing 10% fetal bovine serum (FBS) in a 5% CO₂ incubator. The conjunctival epithelial cells were maintained in Keratinocyte Serum-Free Growth Medium supplemented with 0.05 mg/mL bovine pituitary extract (BPE), 5 ng/mL epidermal growth factor (EGF), 500 ng/mL hydrocortisone, and 0.005 mg/mL insulin at 37 °C in a 5% CO₂ incubator. The corneal epithelial cells were seeded at 5 × 10⁴ cells/well in a 24-well plate and at 1 × 10⁵ cells/well in a 6-well plate. The conjunctival epithelial cells were seeded at 2 × 10⁴ cells/well in a 24-well plate and at 5 × 10⁴ cells/well in a 6-well plate. UPM in PBS was mixed just before treatment.

CCK-8 assay

Cell viability was assessed using Cell Counting Kit-8 (CCK-8) assay kit (Dojindo Molecular Technologies, Rockville, MD, USA). The cells were incubated with or without different concentrations of UPM (10, 50, 100, 300, or 500 µg/mL) for 24 h. The results of CCK-8 assay were determined by measuring the absorbance at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Fluorescence-activated cell sorting (FACS)

At 24 h after UPM exposure, harvested cells were treated with RNase A (200 µg/mL, Sigma-Aldrich) for 1 h and stained with propidium iodide (20 µg/mL, Sigma-Aldrich). The cell cycle was analyzed by measuring the DNA content using LSRFortessa X-20 (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot

The protein was extracted from cells at 24 h after exposure to UPM. An equal amount of protein was loaded onto a SDS-PAGE gel and transferred to a PVDF membrane. The membrane was blocked for 1 h and reacted overnight at 4 °C with specific primary antibodies such as Mucin-1 (Invitrogen, Carlsbad, CA, USA) and Mucin-4 (Invitrogen). Then, the membrane was incubated with the secondary antibodies (Horseradish peroxidase, Cell Signaling, Danvers, MA, USA). Immunoreactive proteins were detected using LAS 4000.

Enzyme-linked immunosorbent assay (ELISA)

At 24 h after UPM treatment, cell culture media was collected and centrifuged to eliminate the cell debris. The levels of inflammatory mediators, such as interleukin (IL)-6 and IL-8, were determined using ELISA kits according to the manufacturer's protocols (Abcam, Cambridge, UK). The absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Inc.).

Fluorescence microscopy

The cells were exposed to UPM for 0–6 h. The cells were incubated with 25 mM carboxy-2'-7'-dichlorodihydrofluorescein diacetate (DCF-DA) in HBSS buffer at 37 °C for 30 min in a 5% CO₂ incubator. Then, cells

were visualized using a laser scanning confocal microscope (FV10i, Olympus, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by multiple comparison tests, between three or more groups using Prism 7.0 software (GraphPad, San Diego, CA, USA). Additionally, we utilized the Student's *t* test for comparison between two groups. Statistical differences were considered significant when $p < 0.05$ or < 0.01 .

Results

Effect of UPM on cell viability in corneal and conjunctival epithelial cells

To investigate the effect of UPM on cell viability, we performed CCK-8 assay in corneal and conjunctival epithelial cells. In corneal epithelial cells, the cell viability in UPM-treated group [10 ($94 \pm 1.3\%$), 50 ($74 \pm 1.1\%$), and 100 $\mu\text{g/mL}$ ($62 \pm 0.4\%$)] was significantly decreased in a dose-dependent manner (Fig. 1a). Consistent with these observations, the cell viability in UPM-treated group [100 ($82 \pm 0.6\%$), 300 ($70 \pm 2.0\%$), and 500 $\mu\text{g/mL}$ ($60 \pm 0.9\%$)] was significantly reduced in a dose-dependent manner (Fig. 1b) in conjunctival epithelial cells.

To clarify the mechanism of toxicity by UPM, we selected the concentration that lowered cell viability to around 60%. Therefore, we treated corneal epithelial cells with 100 $\mu\text{g/mL}$ UPM, and conjunctival epithelial cells with at 500 $\mu\text{g/mL}$ UPM.

Fig. 1 The effect of UPM on cell viability in corneal and conjunctival epithelial cells. Cell viability was observed at 24 h after exposure to UPM. Corneal epithelial cells were exposed to UPM (10, 50, or 100 $\mu\text{g/mL}$). Conjunctival epithelial cells were exposed to UPM (100, 300, or 500 $\mu\text{g/mL}$). **a** The cell viability of corneal epithelial

Effect of UPM on corneal and conjunctival epithelial cell cycle

To investigate the effect of UPM on cell cycle alterations, we performed FACS analysis in corneal and conjunctival epithelial cells. In corneal epithelial cells, the G2/M phase (24.1%) was increased by 24 h of exposure to UPM compared to that in the control group (19.7%), whereas the G1/G0 phase (43.1%) was reduced compared to control group (50.7%) (Fig. 2a). In conjunctival epithelial cells, the increase in G2/M phase (25.3%) was also induced after 24 h of exposure to UPM compared to that in the control group (18.4%), whereas the G1/G0 phase (52.1%) was decreased compared to control group (57.6%) (Fig. 2b).

Effect of UPM on mucin in corneal and conjunctival epithelial cells

To examine UPM exposure-induced disruption of mucin, we performed western blotting in corneal and conjunctival epithelial cells. In corneal epithelial cells, the expression of mucin-1 was decreased in UPM-treated group ($36 \pm 8.3\%$) compared to that in the control group (Fig. 3a, b). However, there was no difference in the expression of mucin-4 between control and UPM-treated group ($92 \pm 26.8\%$). The expression of mucin-1 ($76 \pm 17.4\%$) was decreased by UPM in conjunctival epithelial cells, but no change was detected in the level of mucin-4 expression ($109 \pm 23.9\%$; Fig. 4c, d).

Effect of UPM on inflammatory mediators in corneal and conjunctival epithelial cells

To investigate the effect of UPM on regulation of inflammation mediators such as IL-6 and IL-8, we performed ELISA in corneal and conjunctival epithelial cells. In

cells. **b** The cell viability of conjunctival epithelial cells. Cell viability is expressed as the percentage of control. Data are expressed as mean $\pm$ SEM. $**p < 0.01$ vs. Control (CTL). Urban particulate matter (UPM)

Fig. 2 The effect of UPM on cell cycle of corneal and conjunctival epithelial cells. Cell cycle progression was analyzed by flow cytometry at 24 h after exposure to UPM. Corneal epithelial cells were exposed to 100 $\mu\text{g}/\text{mL}$ of UPM. Conjunctival epithelial cells were exposed to 500 $\mu\text{g}/\text{mL}$ of UPM. **a** The representative results of cell cycle analysis by flow cytometry in corneal epithelial cells. **b** The

phase proportions of corneal epithelial cells. **c** The representative results of cell cycle analysis by flow cytometry in conjunctival epithelial cells. **d** The phase proportions of conjunctival epithelial cells. G1 phase (Blue), S phase (Dark yellow), and G2 phase (Green). Control (CTL). Urban particulate matter (UPM)

corneal epithelial cells, the level of IL-6 (48 ± 3.5 pg/mL) was increased and the level of IL-8 (21 ± 0.8 pg/mL) was decreased at 24 h after exposure to UPM compared to that in the control group [IL-6 (14 ± 0.6 pg/mL) and IL-8 (29 ± 0.9 pg/mL)] (Fig. 4a). In conjunctival epithelial cells, the level of IL-6 (1149 ± 65.0 pg/mL) was increased and the level of IL-8 (27 ± 1.4 pg/mL) was decreased at 24 h after exposure to UPM compared to that in the control group [IL-6 (379 ± 7.4 pg/mL) and IL-8 (84 ± 6.3 pg/mL)] (Fig. 4b).

Effect of UPM on ROS in corneal and conjunctival epithelial cells

To investigate whether UPM induced the generation of reactive oxygen species (ROS), we performed a DCF-DA assay in corneal and conjunctival epithelial cells. The intensity of DCF-DA was measured at 0, 2, 4, and 6 h after exposure

to UPM. In corneal epithelial cells, the intensity of DCF-DA was increased at 4 h after UPM exposure (Fig. 5a). The intensity of DCF-DA was increased at 4 h of UPM exposure compared to that in the control group in conjunctival epithelial cells (Fig. 5b).

Discussion

Our findings demonstrate that exposure to UPM induces toxicological effects on corneal and conjunctival epithelial cells.

Atmospheric particulate matter is a central component of air pollution [13]. It is a mixture of solid and liquid materials from industrial emissions, refuse incineration, geological materials, and so on [14]. World Health Organization (WHO) has reported that atmospheric particulate matter has harmful effects on the human body. Especially,

Fig. 3 The effect of UPM on mucin in corneal and conjunctival epithelial cells. **a** The expression of Mucin-1 (MUC-1) and Mucin-4 (MUC-4) was observed 24 h after exposure to UPM. Corneal epithelial cells were exposed to 100 µg/mL of UPM. Conjunctival epithelial cells were exposed to 500 µg/mL of UPM. **b** Representative photographs of MUC1, MUC4, and β-actin expression in corneal epithelial cells. **c** Representative photographs of MUC1, MUC4, and β-actin expression in conjunctival epithelial cells. **d** The quantitative analysis of MUC1 and MUC4 expression in conjunctival epithelial cells. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. Control (CTL), * $p < 0.05$ vs. CTL. Urban particulate matter (UPM)

lial cells. **b** The quantitative analysis of MUC1 and MUC4 expression in corneal epithelial cells. **c** Representative photographs of MUC1, MUC4, and β-actin expression in conjunctival epithelial cells. **d** The quantitative analysis of MUC1 and MUC4 expression in conjunctival epithelial cells. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. Control (CTL), * $p < 0.05$ vs. CTL. Urban particulate matter (UPM)

Fig. 4 The effect of UPM on inflammatory mediators in corneal and conjunctival epithelial cells. The secreted inflammatory mediators were measured 24 h after UPM exposure. Corneal epithelial cells were exposed to 100 µg/mL of UPM. Conjunctival epithelial cells were exposed to 500 µg/mL of UPM. **a** The quantitative analysis of IL-6 and IL-8 release from corneal epithelial cells. **b** The quantitative analysis of IL-6 and IL-8 release from conjunctival epithelial cells. The secreted inflammatory mediators (IL-6 and IL-8) are expressed as pg/mL. Data are expressed as mean ± SEM. ** $p < 0.01$ vs. Control (CTL), Urban particulate matter (UPM)

Fig. 5 The effect of UPM on ROS in corneal and conjunctival epithelial cells. The DCF-DA (green) was detected 0 h, 2 h, 4 h, or 6 h after UPM exposure. Corneal epithelial cells were exposed to 100 $\mu\text{g/mL}$ of UPM. Conjunctival epithelial cells were exposed to 500 $\mu\text{g/mL}$ of UPM. **a** Representative photographs of DCF-DA in corneal epithelial cells. **b** Representative photographs of DCF-DA in conjunctival epithelial cells. Urban particulate matter (UPM)

the people who live in urban areas are exposed to urban particulate matter associated with rapid urbanization. In our previous study [15], we revealed that UPM induced keratoconjunctivitis sicca in *in vivo* and *in vitro* study. The experiments in this study were conducted focusing on the toxicological effect of UPM on the epithelium of eye from cellular and molecular levels following our previous study.

The corneal and conjunctival epithelium of the human eye is constantly exposed to harmful agents including particulate matter in ambient air. The corneal epithelium, the most superficial component of the ocular surface, in eyes is the outermost mechanical barrier to environmental agents including particulate matter [16]. The density of goblet cells in conjunctival epithelium is decreased by exposure to ambient air pollution [17]. The damage of corneal and conjunctival epithelium involves cell death and disturbance of the mucin expression [1]. During pathogenesis of DES, the DNA damage, cell cycle arrest, and apoptosis in epithelial cells are increased [18]. The progression of the cell cycle can be delayed and/or blocked in response to various dysfunctional protein and genotoxic stresses through the G1/S, G2/M, and metaphase-anaphase (M/A) transition checkpoints [19]. In this study, UPM reduced the viability of corneal and conjunctival epithelial cells in a dose-dependent manner. The viability of corneal epithelial cells (100 $\mu\text{g/mL}$ exposure) was similar to that of conjunctival epithelial cells (500 $\mu\text{g/mL}$ exposure). In addition, the population at S and G2 phase of corneal and conjunctival epithelial cells in UPM-treated group was increased compared to that in the control group. These findings reveal

that UPM may affect the checkpoint of G2/M in corneal and conjunctival epithelial cells.

The inflammatory mediators including IL-6 and IL-8 have a critical role in the causes and consequences of DES [18, 20]. Among inflammatory mediators, there have been conflicting findings about the expression of IL-8 induced by particulate matter. Tau and colleagues reported that DEP decreased the expression of IL-8 protein [4]. In contrast, Yoon et al. reported that ambient particulate matter increased the expression of IL-8 protein [21]. In the present study, our results indicated the same trends as the results of Tau and colleagues. The release of IL-8 was decreased by UPM exposure in both corneal and conjunctival epithelial cells. However, the release of IL-6 was increased by UPM exposure in both corneal and conjunctival epithelial cells. IL-8 is an interleukin that has chemotactic activity for polymorphonuclear neutrophils (PMNs) [22]. The exposure of UPM is unable to induce infiltration of PMNs [23]. Therefore, the decrease of IL-8 release is probably because that the levels of IL-8 protein is regulated by infiltration of PMNs [4, 23].

During DES, it is well known that mucins are regulated by inflammatory factors. [24–26]. It is also reported that expression of the transmembrane mucins in the corneal and conjunctival epithelium are altered by inflammatory mediators [27]. The surface of corneal and conjunctival epithelial cells is covered with a tear film, which includes the mucins, a class of heavily *O*-glycosylated glycoproteins, usually divided into gel-forming and transmembrane mucins [28]. There are different types of transmembrane

mucins including MUC1, MUC4, and MUC16 on the surface of corneal and conjunctival cells [28]. Among the transmembrane mucins, particularly, MUC1 and MUC4 are well known type that is affected during DES [29]. It is also reported that mRNA and protein of MUC1 was decreased in DES, and it led to instability of tear film [26, 30]. In addition, MUC4 is a well-known clinical marker in DES-patients and it can accelerate the aggravation of ocular surface integrity [31].

In the present study, MUC1 was changed by UPM in corneal and conjunctival epithelial cells. However, our results indicated that there was no difference of MUC4 expression between control and UPM-treated group. One possible hypothesis explaining no changes in MUC4 expression by UPM is that MUC4 has a different cleavage site from MUC1. MUC1 has *N*-glycosylated SEA (sperm protein, enterokinase and agrin) domain that also contains proteolytic cleavage sites [28]. Unlike MUC1, the proteolytic cleavage site of MUC4 consists of the amino acids Glycine-Asparagine-Proline-Histidine. At 24 h, UPM exposure may not affect the proteolytic cleavage site of MUC4. The other possible hypothesis is that inflammatory mediators released by UPM regulate the expression of MUC1. It is reported that inflammatory mediators in tears of dry eye patients induced MUC1 expression on corneal epithelial cells [27]. It is also reported that gene expression of mucin was induced by inflammatory mediators via direct and indirect mechanisms [32].

The imbalance between generated ROS and the antioxidant system causes the oxidative stress. The oxidative stress induced ocular surface damage, resulting in DES [16, 21]. Previous studies have revealed that UPM induced ROS leading to cell cycle arrest [19] and release of inflammatory factors [33]. Several studies reported that ROS were involved in cell death [4, 21, 34] and mucin disruption [25, 26, 30] induced by particulate matter. In this study, UPM increased ROS in both corneal and conjunctival epithelial cells. Therefore, ROS could be an upregulation mediator of UPM-induced cell death and mucin disruption.

In conclusion, our findings suggest that UPM causes toxicological effects on corneal and conjunctival epithelium by decreasing cell viability, altering cell cycle, disrupting mucin, and regulating inflammatory mediators possibly through ROS. The limitation of this study is that we did not directly prove the causality of each other. We have just presented the association. In further experiments, we will establish this causal relationship.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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