RESEARCH ARTICLE

The acute respiratory exposure by intratracheal instillation of Sprague–Dawley rats with diesel particulate matter induces retinal thickening

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Abstract

Context: Adverse health effects of ambient particulate matter (PM) have been demonstrated in humans, mostly in terms of respiratory and cardiovascular events. However, whether ambient particle could affect the eyes had not been fully revealed.

Objective: This study investigated the effect of acute respiratory exposure to PM on eyes.

Methods: Diesel exhaust product (DEP) of 200 mg/l was given endotracheally in Sprague–Dawley rats for 1 h (n = 12) and compared to normal control (n = 4). We enucleated eyes and histologically evaluated. Immunohistochemical stains for CD34 (Dako, Glostrup, Denmark, 1:50) and Ki-67 (DakoCytomation, Glostrup, Denmark, 1:150) were performed to evaluate new vessel formation and proliferation activity.

Results: After endotracheal DEP exposure, the thickness of retina was significantly increased to 258 ± 96 μm in DEP group, while that of control was 113 ± 9 μm (p = 0.025). Among the retinal structure, inner plexiform, inner and outer nuclear and rod/cone cell layers were significantly thickened (p = 0.00, 0.017, 0.004, 0.001, respectively). The outer plexiform layer of DEP group showed a tendency of thickening, but statistically insignificant. The afferent fiber and ganglion cell layer showed no thickness difference between two groups, but prominent capillaries with congestion were noted in DEP group. Neither neovascularization nor increased proliferation was demonstrated on CD34 and Ki-67 immunohistochemical staining in DEP group.

Conclusion: This study shows that the acute respiratory exposure of ambient PM increased retinal thickness, especially inner plexiform, inner and outer nuclear and rod/cone cell layers. We thought that increase of retinal thickening in DEP group resulted in hypoxia-induced edema.

Introduction

Progressive industrialization and the increase in the number of motorized vehicles have resulted in high levels of air pollution. Particulate air pollution is an environmental health risk factor that is associated with increased cardiovascular morbidity and mortality. Nowadays, there has been an upsurge of interest in the detrimental effects of pollution and environmental toxins. Ambient ultrafine particles are the most abundant particles in urban environments. A large portion of ambient ultrafine particles is composed of diesel exhaust product (DEP). Ambient particulate matter (PM) is composed of fine particles (diameter ≤2.5 μm) and ultrafine particles (diameter < 0.1 μm) and the common constituents of PM includes nitrates, sulfates, elemental and organic carbon, organic compounds (e.g. polycyclic aromatic hydrocarbons (PAHs)), biological compounds (e.g. endotoxin, cell fragments) and a variety of metals (e.g. iron, copper, nickel, zinc and vanadium)¹. Ambient ultrafine particles remain airborne for long periods of time², penetrate deeply into the respiratory tract and can carry large amounts of toxic compounds, such as hydrocarbons and metals on their surfaces. Both the size and composition of PM are relevant to resultant cardiopulmonary toxicity. Several experimental studies have demonstrated that oxidative stress following exposure to elevated levels of ambient particles leads to activation of proinflammatory reaction and pulmonary effect³,⁴. Adverse health effects of ambient air pollution have been well demonstrated in humans, mostly in terms of respiratory and cardiovascular events⁵–⁸. Several studies demonstrated that DEP effects on cornea and conjunctiva⁹–¹². Exposure to DEP increases inflammatory factor expression in the human conjunctiva and thereby contributes to allergic conjunctival responses¹¹. Human corneal and conjunctival epithelial cells incubated with DEP showed cytotoxicity and an inflammatory response mediated by interleukin-6 (IL-6), but not by tumour necrosis factor-α (TNF-α) or IL-8. The decrease in mucin expression of the cornea cells might leave exposed areas of the cornea to contact with DEP¹⁰. A positive and significant association between exposure to air pollution and goblet cell hyperplasia in human conjunctiva was detected¹². Finally, the increase in mucin expression in the conjunctiva cells might be involved at least in the clearance of DEP to protect the ocular epithelium¹⁰. However, previous studies are
limited on the ocular surface leaving the adverse effects on retina unknown. Retina, which is the most important structure for visual activity, is susceptible to hypoxia and inflammation. Because ambient PM showed cytotoxicity and an inflammatory response, we hypothesized that ambient PM has adverse effects on the retina. To prove this hypothesis, this study evaluated the histological change of retina and other ocular structure after acute respiratory exposure of DEP. Moreover, the neovascularization and proliferation activities of inner and outer nuclear layers of retina were evaluated.

Materials and methods
This study protocol was approved by the Institutional Animal Care and Use Committee of Eulji and Yonsei University College of Medicine and Cardiovascular Research Institute and conformed to the guideline for the care and use of laboratory animals published by the United States National Institutes of Health.

The source and component of DEP
We used commercially available DEP (SRM1650b, National Institute of Standards Technology, Gaithersburg, MD). The certificate of analysis of DEP is presented as a supplementary. Certified concentration values, expressed as mass fractions, for 30 PAHs and 6 nitro-substituted PAH (nitro-PAHs) are provided in Tables 1 and 2 of supplementary material, respectively. Briefly, naphthalene, methylnaphthalene, phenanthrene, methylphenanthrene, benzanthracene and benzo[ghi]perylene were common PAH. Particle size distribution measurements for SRM1650b were carried out using a laser diffraction instrument (Mastersizer 2000, Malvern Instruments, Southborough, MA) and the liquid suspension method with the instrument manufacturer’s small volume liquid suspension dispersion unit (Hydro 2000 SM). The mean particle diameter was 0.18 μm (range 0.12–0.33 μm) with a surface area of 108 m²/g.

In vivo exposure of DEP
Adult male Sprague–Dawley rats (250–300 g) were anesthetized with intraperitoneal injection of ketamine (80 mg/kg) and xylazine (4 mg/kg). After endotracheal intubation and mechanical ventilation (room air, rate 60 cycles/min, tidal volume 1 ml per 100 g of body weight, Harvard Apparatus Rodent Ventilator, model 683), ECG lead II was recorded continuously. In 12 rats (DEP group), DEP dissolved in 0.1 ml phosphate buffered saline (PBS) was given via endotracheal intubation at the concentrations of 200 μg/ml for 1 h. The lung burden of DEP per rat was 20 μg at the DEP concentrations of 200 μg/ml, respectively. For the control (n = 4), the same amounts of PBS were given via endotracheal intubation, respectively. The rat was rapidly sacrificed at the excision of hearts.

Eye extraction and histological examination
Extracted eyes were totally fixed overnight in 10% neutral buffered formalin. Then, eyes were coronally bisected in the middle. We made paraffin embedded tissue blocks according to the conventional methods and sectioned in 4 μm thickness. The sections were stained with hematoxylin and eosin and examined by light microscope. We investigate any histologic differences of cornea, conjunctiva, lens, retina and sclera of both groups. Then, we took pictures of the retina and measured the thickness of each layer including, afferent fibers, inner plexiform, inner nuclear, outer plexiform, outer nuclear and rod/cone cell using ImageJ 1.48p Software (National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/).

Immunohistochemical staining
Immunohistochemical staining was performed using Dako Autostainer plus (DakoCytomation, Carpinteria, CA). Four-micron sections were cut and positioned on poly-L-lysine slides. After deparaffinization and rehydration, antigen retrieval was performed using citrate buffer solution (pH 6.0) at 121 °C for 10 min. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min, then the sections were incubated with specific antibodies against CD34 (Dako, Glostrup, Denmark, 1:50) and Ki-67 (DakoCytomation, Glostrup, Denmark, 1:150). The slides were stained with chromogen 3, 3’-diaminobenzidine and then counterstained with hematoxylin.

Statistical measurements
Data were expressed as the mean ± SEM. Student’s t tests with Bonferroni’s correction were used to compare the means of two numeric values. p Values <0.05 were considered statistically significant.

Results
Retinal thickening after acute exposure of DEP
The most striking finding of ocular change after acute DEP exposure is retinal thickening. Figure 1 shows the typical example of the retina in control and DEP exposure group. The whole thickness of retina was significantly increased to 258 ± 96 μm in DEP group while that of control was 113 ± 9 μm (p = 0.025) (Table 1). Compared with control, capillaries of the afferent fiber layer of DEP group were markedly dilated and showed compact RBCs (arrow). This finding is compatible with capillary congestion because of edema (Figure 2).

The thickness of each layer of the retina was summarized in Table 1. Especially inner plexiform (20 ± 4 versus 71 ± 18 μm, p < 0.001), inner nuclear (9 ± 1 versus 28 ± 2 μm, p < 0.001), outer nuclear (28 ± 2 versus 76 ± 23 μm, p = 0.004) and rod/cone cell layer (16 ± 5 versus 55 ± 16 μm, p = 0.001) were significantly increased after the acute exposure of DEP. Compared with control, the thickness of the outer plexiform layer of DEP group showed the trend of increase, but statistically insignificant (4 ± 1 versus 43 ± 94 μm, p = 0.495). However, there was no significant change after DEP exposure in afferent fiber and ganglion cell layer.

Other ocular change after acute exposure of DEP
Figure 3 shows the infiltration of neutrophils (arrows) at the superficial cornea. This finding was observed in 2 (17%) out
Figure 1. Microscopic findings of the retina from (A) control and (B) diesel exhaust product (DEP) group. DEP group shows marked thickening of inner plexiform (IPL), outer nuclear (ONL) and rod/cone cell layer (RCL) (H&E, 400×). AFL indicates afferent fiber layer; INL, inner nuclear layer; OPL, outer plexiform layer.

Table 1. The comparison of thickness between two groups.

| Thickness                  | Control (n = 4) | DEP (n = 12) | p value |
|----------------------------|----------------|--------------|---------|
| Whole layer                | 113 ± 9        | 258 ± 96     | 0.025   |
| Afferent fiber layer       | 34 ± 12        | 32 ± 11      | 0.867   |
| Inner plexiform layer      | 20 ± 4         | 71 ± 18      | <0.001  |
| Inner nuclear layer        | 9 ± 1          | 50 ± 25      | 0.017   |
| Outer plexiform layer      | 4 ± 1          | 43 ± 94      | 0.495   |
| Outer nuclear layer        | 28 ± 2         | 76 ± 23      | 0.004   |
| Rod/cone cell layer        | 16 ± 5         | 55 ± 16      | 0.001   |

12 cases of DEP group, but none in control. Figure 4 shows the change of cornea and conjunctiva after DEP exposure. Compared with control (Figure 4A), there was no significant difference in thickness of cornea in DEP group (Figure 4B). Conjunctiva also showed no significant difference between control (Figure 4C) and DEP group (Figure 4D). Sclera and lens also showed no change after the exposure to DEP (data not presented).

Results of immunohistchemical stain

To evaluate whether the prominent capillaries of retinal afferent fiber layer in DEP group were related with new vessel formation, we performed CD34 immunostaining. However, CD34 immunostaining was negative in DEP group (Figure 5A). This finding suggests that the prominent capillaries of retinal afferent fiber layer in DEP groups were caused by capillary congestion rather than neovascularization.

Moreover, Ki-67 immunostaining for mitotic activity was performed to evaluate the inner and outer nuclear layer thickening caused by cell proliferation. Ki-67 immunostaining for mitotic activity was negative in DEP group (Figure 5B). This result suggests that the inner and outer nuclear layer thickening in DEP groups were caused by edema rather than cell proliferation.

Discussion

The main finding of this study is that the acute respiratory exposure of ambient PM increased retinal thickness, especially inner plexiform, inner and outer nuclear and rod/cone cell layers. Second, there was no significant change in cornea and conjunctiva. Finally, because CD34 and Ki-67 immunostaining of inner and outer nuclear layer was negative, the thickening of the retina was not related with neovascularization and cell proliferation. These findings suggest that the retinal thickening was related with hypoxia-induced edema. Most published works described observations of local effects through local eye contact with ambient air or test material (or cells in suspension)\textsuperscript{9,11}. To our knowledge, this is the first study describing the observed local effects in the eye after systemic exposure through the lungs.
Hypoxia-induced edema of retina by acute exposure of DEP

In this study, we histologically observed marked retinal thickening in DEP group and there was no evidence of new vessel formation or proliferation activity. Respiratory exposure of ambient PM declines pulmonary and cardiovascular functions and cause systemic hypoxia. Hypoxia increases vascular permeability in neural tissues such as the brain and retina and induces damage to or loss of retinal ganglion cells in the developing retina with resulting visual impairment. Hypoxia of retina affected the integrity of the tight junction through a reduced expression of tight junction-associated proteins, like zona occuldens-1 (ZO-1) and occludin, resulting in increased blood-retinal barrier permeability leading to vasogenic edema in the adult rat retina\textsuperscript{13}. Kaur et al.\textsuperscript{14} reported that the hypoxic animals showed the multivesicular...
aggregations in the lumen of retinal capillaries. The multi-vesicular aggregation might obstruct the capillary lumen and sluggish blood flow enhancing the retinal hypoxia. Consistently, our result also showed prominent capillary of the retina in DEP group. Moreover, an increased production of factors such as vascular endothelial growth factor, nitric oxide, inflammatory cytokines and free radicals has been implicated in this process14,15.

The fluid accumulation contributes to the degeneration of retinal neurons and to a decrease in visual acuity, as it results in compression of neurons, nerve fibers and vessels (which exacerbates ischemic conditions), and in an elongation of the routes for the diffusion of metabolic substrates and oxygen16. Prolonged retinal edema, especially involving macular area, result in impaired visual acuity. Since the burden of air pollution on health is significant at even relatively low concentrations, efforts that reduce fine PM air pollution is necessary to reduce health risks to a minimum.

Other ocular change by acute exposure of DEP

In this study, we observed mild neutrophil infiltration in the cornea in DEP group. DEP was reported to induce cytokine expression in human bronchial epithelial cells and cardiomyocytes17–19. Several studies reported that the exposure to DEP increases inflammatory factor expression in the human conjunctiva and thereby contributes to allergic conjunctival responses via increasing cytotoxicity and an inflammatory response mediated by upregulated expression of intercellular adhesion molecule 1 and IL-69,10. Several studies reported that chronically affected eyes by DEF showed thickening of eyelids, eyelid margin vascularization or hyperkeratinization and even loss of eyelashes20,21. The suggested mechanisms were goblet cell hyperplasia11 and meibomian gland dysfunction12. Although mild corneal inflammation was observed in two cases of DEP group, we interpreted it as a localized inflammation independent of DEP exposure. As a result, acute exposure of DEP affects only the retina, not any other ocular structures including the eyelid, conjunctiva or lens.

Study limitations

Inhaled nanosized particles in air pollution can be transmigrated across human pulmonary epithelium into the systemic arterial circulation22. However, there is no data on the actual concentration of ultrafine particles in the blood after inhalation. Therefore, it is hard to tell the critical concentration of ultrafine particles that can affect the retina. The ultrafine particle is much higher in our study than the physiologic condition. Further study would be necessary to determine the long-term effect of ambient PM exposure on eyes. Finally, the direct causality of systemically induced local hypoxia through the instillation of DEP is not certain in this study. It might be indirect.

Conclusion

After acute respiratory exposure of DEP, all of the retinal structures except innermost afferent fiber layer were thickened. Prominent capillaries with congestion were observed in the afferent fiber layer. However, neovascularization or proliferation activity was not observed. These findings suggest that respiratory exposure of ambient PM can induce hypoxia associated retinal thickening. To our knowledge, this is the first study describing the observed local effects in the eye after systemic exposure through the lungs.

Declaration of interest

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Supplementary material available online