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Effects of ambient levels of traffic-derived air pollution on the ocular surface: Analysis of symptoms, conjunctival goblet cell count and mucin 5AC gene expression☆

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ABSTRACT

Purpose: To quantify ocular symptoms, goblet cells (GC) and mucin 5AC (MUC5AC) gene expression on the conjunctiva of healthy subjects exposed to ambient levels of traffic-derived air pollution and to estimate its correlation with NO_2 and particulate matter smaller than 2.5 μm ($PM_{2.5}$) levels.

Methods: Twenty-one taxi drivers or traffic controllers were assessed with the Ocular Surface Disease Index (OSDI) questionnaire and conjunctival impression cytology. MUC5AC mRNA levels were determined based on the cytology of the right eye, and GC density was assessed based on the cytology of the left eye. Mean individual levels of 24-h NO_2 and $PM_{2.5}$ exposure were assessed the day before examination. Possible associations between NO_2 or $PM_{2.5}$ levels, OSDI scores, GC densities and MUC5AC mRNA levels were verified.

Results: The subjects were exposed to mean $PM_{2.5}$ levels of 35 ± 12 μg/m^3 and mean NO_2 levels of 189 ± 47 μg/m^3. OSDI scores were low (7.4 ± 8) and GC densities were 521 ± 257 and 782 ± 322 cell/mm^2 on the bulbar and tarsal conjunctivas, respectively. The mean GC-derived MUC5AC mRNA expression was 14 ± 7 fM/μg of total RNA. A significant and positive correlation was observed between MUC5AC mRNA levels and tarsal GC density ($p=0.018$). A trend toward association between $PM_{2.5}$ levels and tarsal GC cell density ($p=0.052$) was found.

Conclusion: Exposure to ambient levels of air pollution impacts conjunctival GC density. An increase in MUC5AC mRNA levels may be part of an adaptive ocular surface response to long-term exposure to air pollution.

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1. Introduction

Adverse health effects of ambient levels of air pollution have been demonstrated in humans, mostly in the form of respiratory and cardiovascular events (Kunzli et al., 2001; Medina-Ramon et al., 2006; Samoli et al., 2003). However, few studies have investigated the effects of air pollution on the ocular surface despite the fact that the eye is continuously exposed to the external environment (Torricelli et al., 2011; Wolkoff, 2010). In fact, ocular symptoms were associated with an ambient air pollution exposure (Novaes et al., 2011; Saxena et al., 2003; Versura et al., 1999) and previous studies have documented air pollution-related ocular surface abnormalities as assessed with the tear film break-up test, the Schirmer test (Gupta et al., 2002; Saxena et al., 2003), lysozyme activity (Gupta et al., 2002) and osmolarity assays (Torricelli et al., 2013).

Ambient air pollution is a complex mixture of particulate matter (solid and liquid particles suspended in air) and gases such as ozone, nitrogen dioxide (NO_2) and carbon monoxide. Traffic-related air pollution is commonly assessed by particulate matter (PM) and NO_2 levels (Grigg, 2012; Jerrett et al., 2009). Although NO_2 can be generated when oxygen or ozone in the air oxidizes nitrogen monoxide, in outdoor air, the main source of NO_2 is fuel combustion, primarily from motor vehicles, and in addition, from power station and factories (Kelly et al., 2012). The main source of

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PM in urban areas is road transport in addition to the burning fuels in power stations and factories as well. Components of traffic-derived PM are engine emissions, brake and tire wears, and dust from road surfaces (Kelly et al., 2011).

Impression cytology is another important, simple and non-invasive technique for investigating a number of ocular surface disorders (Singh et al., 2005). One study using impression cytology reported a positive association between exposure to air pollution and goblet cell (GC) hyperplasia in human conjunctiva (Novaes et al., 2007). Another study (Versura et al., 1999) demonstrated an increase of inflammatory cells on the ocular surface related to air pollution exposure.

Impression cytology can also be used to collect cells from the ocular surface for assessing ocular mucin mRNA levels in healthy individuals (Argueso et al., 2002; Corrales et al., 2003). An important part of the ocular surface consists of mucins, a type of high-molecular-weight proteins usually divided into two main groups: gel-forming and transmembrane mucins (Caffery et al., 2010; Corrales et al., 2011). On the ocular surface, the large gel-forming mucin 5AC (MUC5AC) is expressed by goblet cells and hydrates in the tear film and acts as a lubricating agent and clearing molecule (Gipson, 2004). Mucins have been implicated in the pathophysiology of dry eye disease (Caffery et al., 2010; Corrales et al., 2011) and many other ocular surface abnormalities, including atopic keratoconjunctivis, vernal keratoconjunctivis (Dogru et al., 2005) and infectious diseases (Kardon et al., 1999). Indeed, gel-forming MUC5AC seems to play a key role in dry eye disease (Argueso et al., 2002). However, to our knowledge, no study has evaluated the relationship between ambient levels of air pollution and ocular surface mucins.

The purpose of the present study was therefore to estimate conjunctival GC density, MUC5AC gene expression, and ocular symptoms in a group of individuals exposed to ambient levels of traffic-derived air pollutions and its possible correlations with NO_2 and particulate matter smaller than 2.5 μm ($PM_{2.5}$) levels.

2. Materials and methods

2.1. Study population

Approval from the Institutional Review Board was obtained for the study. The study followed the principles of the Declaration of Helsinki and informed consent was obtained from all participants.

The study involved healthy male volunteers working as taxi drivers or traffic controllers in São Paulo, Brazil, one of the largest and most polluted cities in Latin America (de Toledo and Nardocci, 2011). The volunteers included in the study were required to have lived in the study area for at least 5 years and to sign an informed consent form. The exclusion criteria were smoking, inability to comply with the schedule of visits of the study protocol, wearing contact lenses, previous ophthalmic surgery and preexisting ophthalmic diseases or use of eye drops. In addition, no subject was taking any oral medication (e.g. anti-hypertensive, antidepressants, and diuretic medications).

2.2. Air pollution exposure assessment

NO_2 and $PM_{2.5}$ were used as indicators of exposure to traffic-derived air pollution. We employed a monitoring system composed of NO_2 passive tubes as previously described (Novaes et al., 2007). Briefly, the subjects carried a passive sampler fitted with qa cellulose filter (Energetica, Rio de Janeiro, Brazil) impregnated with an absorbent solution containing 2% triethanolamine, 0.05% *o*-methoxyphenol and 0.025% sodium metabisulfite inside a small plastic tube with one of its extremities open to ambient air. The nitrite produced during sampling was determined calorimetrically by reacting the exposed absorbing reagent with sulfanilamide and 8-anilino-1-naphthalene-sulfonic acid (ANSA) at a wavelength of 550 nm. The method was found to have an accuracy of 98.6% and a precision of 80% (Novaes et al., 2007). Particulate matter was sampled with a personal gravimetric particulate impactor as described by Lee (2005) with slight modifications. Briefly, a portable sampling pump provides a constant 1.8 L/min flow rate for 24 h and draws ambient air through the impactor. The $PM_{2.5}$ is retained by a 37-mm teflon membrane and the particles suspended in air are measured gravimetrically. Quality assurance and quality control (QA/QC) procedure included the determination of the limit of detection (0.47 μm/m^3), the precision (0.42 μm/m^3), and the coefficient of variation (0.03) (Lee et al., 2005).

The subjects were instructed to carry the samplers at all times during their daily activities in a regular day of work and to place them by the bedside at night (monitoring was carried out for 24 h, starting at 8 a.m.).

2.3. Ocular Surface Disease Index questionnaire

The subjects were evaluated using the Ocular Surface Disease Index (OSDI) questionnaire (Schiffman et al., 2000), translated into Portuguese (Prigol et al., 2012), containing 12 questions for the assessment of the presence or absence of ocular dryness, irritation, heaviness, fatigue and itching over the preceding 7 days. The test scale ranged from 0 to 100, with higher scores representing greater disability. In all subjects, the OSDI questionnaire was applied in the Laboratory for Investigation in Ophthalmology, at the University of Sao Paulo Medical School, in the same morning (11:00–12:00 am) on which air pollutant samplers were removed. Then, conjunctival goblet cell samples were collected. Ambient temperature and air humidity were monitored during the study period.

2.4. Conjunctival goblet cell assays

Impression cytology was used to obtain samples from the superficial epithelial cell layers of the conjunctiva. Briefly, semicircular filters of approximately 15 mm diameter in size (cellulose ester filter 22-μm pore; Millipore Corp., Bedford, MA, USA) were applied to both temporal bulbar and temporal–inferior tarsal conjunctiva after instillation of one drop of topical anesthetic (0.5% tetracaine, FMUSP, Brazil) in the left eye. The paper fragments were applied for approximately 10 s, and after gentle pressure with the blunt end of the forceps, the fragments were peeled off and immediately immersed in tubes containing absolute ethanol. After fixation, the specimens were rehydrated in 70% ethyl alcohol, then placed successively in alcian blue reagent, periodic acid-Schiff reagent, sodium metabisulfite, Gill's hematoxylin and Scott's tap water. The specimens were then rinsed with 95% alcohol and absolute alcohol. Xylene was used to make the filter paper transparent. Before mounting, the filter paper was placed with the epithelial cells facing up. The slides were examined under light microscopy and the goblet cells were counted in 13 high-power fields at 400 × magnification (Singh et al., 2005).

2.5. MUC5AC Assays

Impression cytology was also performed on the inferior–tarsal conjunctiva of the right eye, after which the filter paper was placed in an empty sterile tube for subsequent total mRNA extraction. All samples were immediately placed on ice and then stored at −80 °C until processing.

2.5.1. RNA isolation, reverse transcription, and real-time polymerase chain reaction

Total RNA from impression cytology samples was isolated using an RNA extraction kit (RNeasy Mini Kit, Qiagen) and cDNAs generated from 50 ng total RNA were obtained using a reverse transcriptase kit (SuperScript II Reverse Transcriptase Kit, Invitrogen) (Corrales et al., 2011). The absolute mRNA concentration of MUC5AC was determined for 300 ng cDNA using real-time polymerase chain reaction (RT-PCR) and submitting samples to a 40-cycle amplification according to the instructions of the manufacturer of the PCR kit (Rotor-Gene SYBR Green PCR Kit, Qiagen). RT-PCR reactions were performed in triplicate for each sample in a total volume of 25 μL. The following primers were used in the study: 5′-(TCCACCATATACCGCCACAGA)-3′ (sense) and 5′-(TGGACCGACAGTCACTGTCAAC)-3′ (antisense) for MUC5AC; 5′-(GAAGGTGAAGGTCGGAGTC)-3′ (sense) and 5′-(GAAGATGGTGATGGGATTTC)-3′ (antisense) for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Argueso et al., 2002). The average threshold cycle (CT) values for GAPDH were used as an endogenous reference.

To obtain the MUC5AC standard curve, the amplification reaction band of MUC5AC was purified from 1% low melting agarose using a QIAquick kit (Qiagen). The transcript concentration was determined by absorbance measurement using a spectrophotometer (Nanodrop 2000c, Thermo Scientific) and a standard curve of MUC5AC was drawn based on serial dilutions from 10^6 to 0.1 fg. The resulting curve presented a slope of −3.38 (r=0.99) indicating optimal efficiency for the RT-PCR assay. Finally, unknown MUC5AC mRNA was quantified from the initial standard curve of MUC5AC using the Rotor-Gene ScreenClust HRM software (Qiagen).

2.6. Statistical analysis

Quantitative variables were expressed in terms of central tendency and dispersion. The normal distribution of the variables was verified with the Skewness–kurtosis test. Correlations among NO_2 or $PM_{2.5}$ levels, OSDI questionnaire scores, GC densities and MUC5AC mRNA levels were investigated using Spearman or Pearson's correlation. The level of statistical significance was set at 5%.

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