The effect of air pollutants on the microecology of the respiratory tract of rats⋆

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ABSTRACT

To study the effect of air pollution on the microecology of the respiratory tracts and the relationship of the biotopes with respiratory diseases, Wistar rats exposed to mixed air pollutants were used as poisoning models. The bacterial floras of respiratory tract were analyzed as well as expression of pro-inflammatory mediators of the respiratory epithelium. The mRNA and protein expression levels of pro-inflammatory factor and cytokines measured showed that there were significant changes in the microbiocenosis of the respiratory tract. The microorganisms underwent quantitative and qualitative changes following exposure to mixed air pollutants including a decline of indigenous microflora and increase of the content of conditionally pathogenic microorganisms. These changes depended on the degree of air pollution severity. Measurement of pro-inflammatory factors CC16, TNF-α and IL-6 revealed a similar time-dependent relationship between the content of conditionally pathogenic microorganisms and the interference of CC16 secretion, as well as up-expression of TNF-α and IL-6.

1. Introduction

Air pollution has increased with development of modern industry, dense population of cities, and rapid increase in the use of fossil fuels. Increasing epidemiologic evidence has suggested strong correlation between respiratory disease and atmospheric pollution (Atkinson et al., 2001; Calderon-Garciduenas et al., 2007; Chapman et al., 1997; Dockery and Pope, 1994; Epton et al., 2008; Vigotti et al., 2007). At present, air pollutants are released at the rate of approximately 600 million tons-per-year worldwide resulting in a dramatic increase in the number of hospitalizations, physician visits, school absences, and asthmatic episodes that associated with elevated levels of particulate matter (PM) or ozone in urban settings (Bascom and Kesavanathan, 1997; Calderon-Garciduenas et al., 2007; Fahmy et al., 2010; Grigg, 2009; Harrod et al., 2005; Plopper et al., 2007; Zanobetti et al., 2000).

The respiratory tract functions as the major gas exchange interface in humans. The normal bacterial flora of the respiratory tract acts as an important host barrier contributing to host defense against other pathogenic bacteria (Cangemi de

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Gutierrez et al., 1999, 2001; Reynolds, 1987). A dynamic balance exists between the host and commensal normal bacteria floras to mitigate the effects of potentially harmful environmental factors. Any bacteria exceeding capacity threshold can damage the microecological balance and lead host disease. *Prevotella*, the Gram-negative bacteria is recognized as part of the oral and vaginal flora, and is detected from respiratory tract infections and in the lungs of children with cystic fibrosis.

Clara cells are a kind of epithelial cell without cilia and are found in the bronchial mucosa capable of secreting proteins to perform important physiological functions (Ficker, 2008; Rubin, 2002; Wong et al., 2009). As one of the most primary secretory proteins of Clara cells, Clara cell secretory protein (CC16) has a low-molecular weight protein of 16 kDa and is mainly secreted by non-ciliated bronchiolar Clara cells in large amounts into the lumen of the respiratory tract. It has been reported that CC16 exhibits immunosuppressive and anti-inflammatory properties, which can supress the synthesis of proinflammatory cytokines in bronchoalveolar lavage fluid (BALF). Thus, CC16 expression in BALF has been proposed to be an important marker to measure the degree of lung injury.

Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) are known as important pro-inflammatory factors that help to regulate the inflammatory process in response to bacteria and other pathogens (Strieter et al., 2002). TNF-α is an important initiation factor for inflammation, and is capable of modulating the discharge of IL-1 and IL-6 cytokines (Wu and Zhou, 2010). IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T-cells and macrophages to stimulate immune response.

Increasing lines of evidence have shown that the normal flora disequilibrium may be involved in the process of infection, many cytokines and secretory proteins (including TNF-α, IL-6) play a key role in evaluating the degree of inflammation and reducing the damage of reaction, there are rarely reported to link the respiratory illness with both the air pollution and normal flora aberration. The aim of our study was to simulate real atmospheric pollution by mixing the air pollutants with a standard air mixture, and then exposing to animal model. We try to demonstrate the effects of air pollutants on the microecology in the respiratory tract, to investigate the association of the air pollution and normal flora aberration with the respiratory system diseases and to explore the regulation mechanisms of related molecules.

2. Methods

2.1. Preparation of PM$_{10}$ suspended liquid and mixed air pollutants

PM$_{10}$ was collected continuously at a flow rate of 20–30 L/min from areas of coal burning, traffic congestion in Shenyang city (China) by a PM$_{10}$ air sampler (KB-120F, Suzhou Ovbei Co, LTD) for two months. Total 300 mg PM$_{10}$ was eluted from fiberglass membranes by ultrasonic vibrator and prepared in 0.9% NaCl solution at different dosages: 22.5 mg/ml (high dose), 15 mg/ml (middle dose) and 7.5 mg/ml (low dose).

A standard air mixture was provided by Dalian Special Gas Industry Co. The concentrations of SO$_2$, NO$_2$, CO were 22.5, 18 and 600 mg/m^3 respectively for the high dose; 15, 12 and 400 mg/m^3 for the middle dose and 7.5, 6 and 200 mg/m^3 for the low dose.

2.2. Animal model

96 Wistar rats (48 males and 48 females, weight range 200–240 g) were purchased from China Medical University Laboratories and were randomly divided into three tested groups (low, middle and high dose) and a control group, each was subdivided into 3 groups (1d, 15d and 30d), 8 rats in each group. Animals received the same food, water and were kept at the same ambient conditions of temperature, humidity and noise. Rats in low, middle, and high dose groups were respectively exposed to PM$_{10}$ of 7.5, 15, and 22.5 mg in 1 ml saline by intratracheal instillation, and then were respectively exposed to mixed gases of SO$_2$ (7.5, 15, and 22.5 mg/m^3), NO$_2$ (6, 12, and 18 mg/m^3), and CO (200, 400, and 600 mg/m^3) by static inhalation 2 h per day for 1d, 15d and 30d respectively. 1 ml saline was instilled and general air was inhaled in rats of control group.

2.3. Isolating and identifying the bacteria from the pharynx of the rats

Following 1d, 15d, and 30d exposure to the mixed air pollutants, the posterior pharyngeal secretions of rats were taken using a microbial-specific sterile cotton swab each time and rapidly collected in 1 ml Ringer's solution with glass beads in sterile glass bottles. 100 µl mixed solution was mixed with 0.9 ml sterile physiological saline to produce a 100-fold dilution. 100 µl diluted solution was then put onto a nutrient blood agar plate, an eosin methylene blue (EMB) plate, and anaerobe blood agar. The nutrient blood agar plate and EMB plate were cultured at 37 °C for 24 h for aerobic culture. The anaerobe blood agar was placed into an anaerobic box at 37 °C for 72 h. By visual inspection, the colony characteristics and colony count were used to identify different colonies which were subsequently isolated and purified with a plate streaking. Preliminary identification of the bacteria was then done by using a Gram stain.

To identify aerobic bacteria, after Gram staining and microscope examination, selected strains of *Staphylococcus aureus*, *Staphylococcus* and *Corynebacterium* bacteria were chosen for biochemical identification with the ID32STAPH, ID32STREP, APICORYNE rapid biochemical identification reaction diagnosis reagent strips respectively. Chosen strains of Gram-negative bacteria were selected for biochemical identification with ID32GN rapid biochemical identification reaction diagnosis reagent strips. All the steps were followed by the manufacturer's instructions. The number of colonies was analyzed by BioMérieux (France) Bacteria Identification and Antibiotic Susceptibility Analysis system ATB.

For the identification of anaerobic bacteria, API 20A anaerobe identification test lip was used following by the manufacturer's instructions and the number of bacteria was analyzed with the France bioMérieux ATB bacterial identification system V3.0.
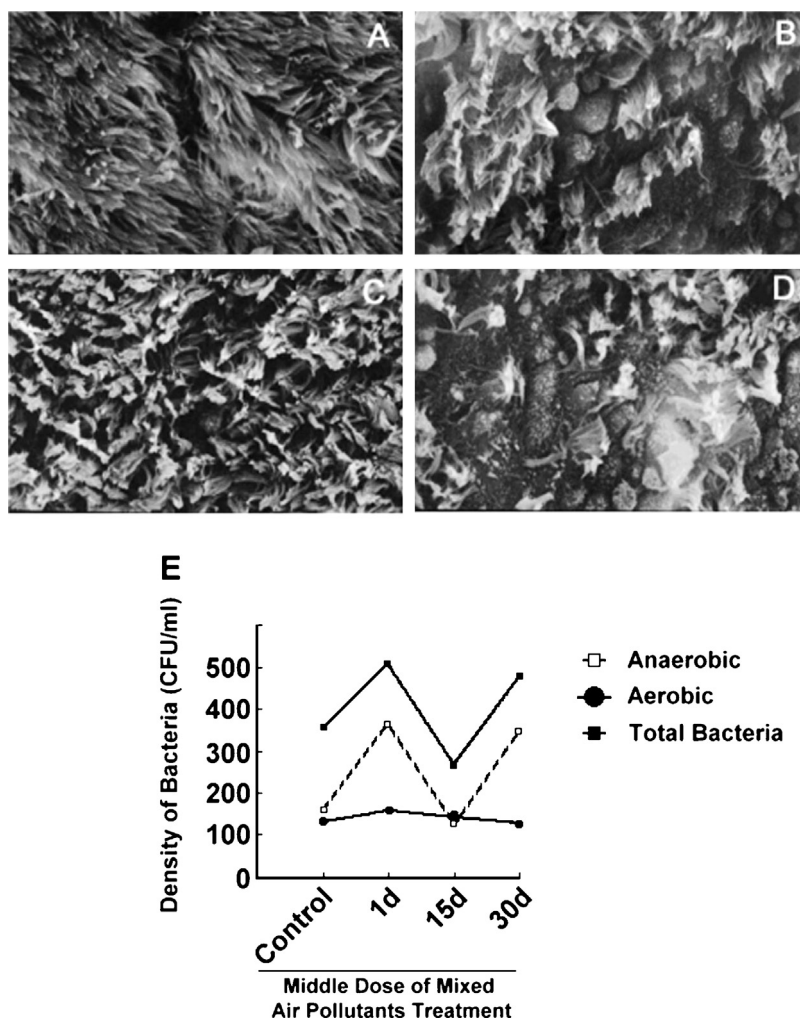
Fig. 1 – Effects of exposure time on oropharyngeal bacterial flora density following the exposure of rats to mixed air pollutants. A–D: SEM images of the changes of oropharyngeal ultrastructure of rats by mixed air pollutants. E: Time sensitive experiment to show the bacterial flora density changes following the exposure of the middle dose of mixed air pollutants. The density of bacteria was represented by CFU/ml.

2.4. Scanning electron microscope

The rat tissues were flushed and perfusion-fixed with 2.5% paraformaldehyde for 2 h at 4 °C. After fixation, tissues intended for scanning electron microscopy were pinned out to expose the luminal surface and fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Tissues were coated with gold–palladium to a thickness of about 15 nm in a Denton DV-502 high-vacuum evaporator. After coating, du Pont conductive silver paste was placed on the tissue surface. These samples were observed in SEM (KYKY-1000B).

2.5. RNA extraction and semi-quantitative real-time RT-PCR

Total RNA of rat lung that treated with middle dose of mixed air pollutants at different time points was isolated using a RNeasy Mini Kit (Qiagen Sciences). 2 μg RNA was reverse-transcribed with SuperScript reverse transcriptase and random primers

(Invitrogen) according to the manufacturer's instructions. Gene expression was quantified using SYBR® Green real-time PCR (Applied Biosystems) and primers were generated by TaKaRa Biotechnology. The real-time PCR conditions were 5 mins at 94 °C, following by 30 cycles of 45 secs at 94 °C and 1 min at 56 °C, followed by a final step of 1 min at 72 °C. The gels were photographed, and the bands were quantified with NIH Image version 1.5 software for Macintosh computers and the results standardized to GAPDH for representation of the level of gene expression. The sequences of primers of CC16, TNF-α and IL-6 were designed as follows:

CC16:	5′-CATCAGCCCACATCTACAGAC-3′
	5′-GGGCTTTAGCGTAGAATATCT-3′
TNF-α:	5′-CATCTGCTGGTACCACCAGTT-3′
	5′-TGAGCACAGAAAGCATGATC-3′
IL-6:	5′-GAGAGCATTGGAAGTTGGGC-3′
	5′-CTTCCAGCCAGTTGCCTTCT-3′
GAPDH:	5′-ACCACAGTCCATGCCATCAC-3′
	5′-TCCACCACCCTGTTGCTGTA-3′
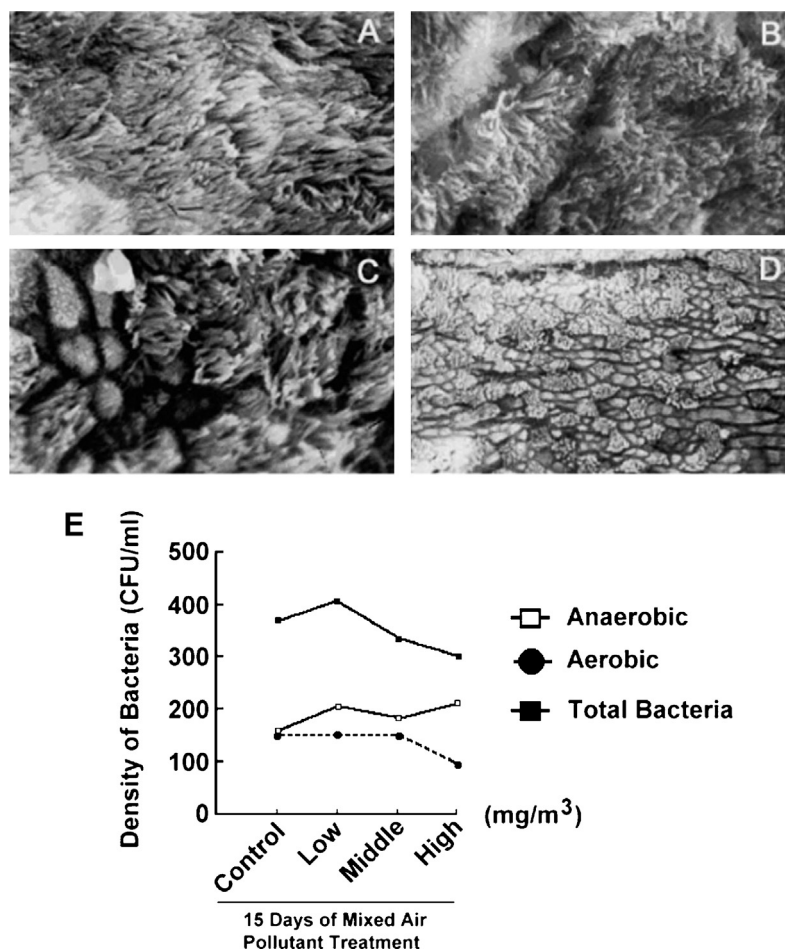
Fig. 2 – Effects of the dose of mixed air pollutants on oropharyngeal bacterial flora density following exposure of the rats to mixed air pollutants. A–D: SEM images of the pathologic changes of oropharynx on different doses of mixed air pollutants. E: Dose sensitive experiment to show the bacterial flora density changes after 15d exposure of mixed air pollutants. The density of bacteria was represented by CFU/ml.

2.6. Western blot

Gathering rat samples that treated with middle dose of mixed air pollutants at different time points. Total protein was extracted using T-PER protein extraction regent (Thermo Scientific) following the manufacturer's instructions. The protein concentration was quantified using the BCA protein assay kit (Thermo Scientific). 20 μg protein was loaded by electrophoresis using 4% Tris gel and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked with blocking buffer with 5% skim milk and 0.1% Tween® 20 (Sigma) for 1 h. The primary antibody used was goat anti-rat CC16 polyclonal antibody (Santa Cruz) and incubated with membrane for 1 h at 4 °C. The membrane was washed with phosphate buffered saline with 0.1% Tween®-20 (PBS-T) at room temperature and incubated with rabbit anti-goat polyclonal antibody conjugated with horseradish peroxidase (HRP) at 4 °C for 1 h. The protein was detected using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposure to Kodak film (Kodak). The density of bands was analyzed using the NIH Image program (http://rsb.info.nih.gov/ij/).

2.7. ELISA

The levels of CC16, TNF-α and IL-6 in BALF were measured using ELISA (Jingmei Biotech) following the manufacturer's instructions. The optical density was measured by an automated microplate ELISA analyzer (CLINIBIO-128).

2.8. Data analysis

All results were analyzed by SPSS10.0 software. Data were presented as mean ± s.e. Values for $p < 0.05$ were considered statistically significant.

3. Results

3.1. The changes in the microbiocenosis of the respiratory tract

3.1.1. Effects of exposure time on oropharyngeal bacterial flora density following the exposure of rats to mixed air pollutants

The changes of oropharyngeal ultrastructure of rats by mixed air pollutants were observed under scanning electron

microscope. The tracheal ciliated columnar epithelium of rats remained dense and organized in control group after exposure to normal air for 30d (Fig. 1A). After exposure to mixed air pollutants in middle dose ranges for 1d, the ciliated columnar epithelium ciliated exhibited disorderly shape, cellular adhesions, and goblet cell hyper-secretion (Fig. 1B). At 15d post-exposure, the cilia became tangle-like adhesions, and goblet cell hyper-secretion was more vigorous. Additionally, there were a significant amount of mixed air pollutants that adhered to the epithelial surface (Fig. 1C). At 30d post-exposure to mid-range mixed air pollutants, cilia became shorter with increasing tangle-like adhesions, and some cilia lost adhesion to the basement membrane causing focal epithelial defects (Fig. 1D).

The bacterial flora analysis demonstrated that oropharyngeal bacterial flora density had significant fluctuations following the exposure of the mixed air pollutants in middle dose at different time points. The anaerobic bacteria density in the oropharynx increased significantly at 1d post-exposure ($p < 0.01$), decreased at 15d post-exposure, and then significantly increased again at 30d post-exposure ($p < 0.01$) (Fig. 1E).

3.1.2. Effects of the dose of mixed air pollutants on oropharyngeal bacterial flora density following exposure of the rats to mixed air pollutants

The pathologic changes of oropharynx on different doses of mixed air pollutants were observed by scanning electron microscopy. Exposure to normal air for 15d, caused no changes to the morphology of tracheal ciliated columnar epithelium which remained dense and well organized in the control group (Fig. 2A). However, in the low dose pollutant treatment group, the cilia of the tracheal ciliated columnar epithelium presented tangle-like adhesions (Fig. 2B). In middle dose treatment group, the adhesion of the cilia to the tracheal ciliated columnar epithelium became more pronounced and there was the appearance of squamous metaplasia (Fig. 2C). In the high dose exposure group, tracheal tissue exhibited a large degree of squamous metaplasia (Fig. 2D).

Bacteria identification showed that low and middle dose treatment of mixed air pollutants had no significant effect on the density of oropharyngeal aerobic bacterial flora, but the densities of aerobic bacteria decreased in high dose treatment group. Although there were fluctuations in anaerobic bacteria at differential doses, the density of total bacteria continued to decline with the increasing of the pollutant doses (Fig. 2E).

3.2. The pro-inflammatory factors or cytokines in the respiratory tract of rats

3.2.1. Effect of mixed air pollutants on CC16 and cytokine mRNA expression in rat lung tissue

The real-time PCR results demonstrate that the mRNA level of CC16 in the rat lung tissue at 1d and 15d were significantly lower than that of the control group ($p < 0.05$), but there was no significant difference in comparison to the 30d group (Fig. 3A). In contrast to CC16 expression, TNF-α mRNA expression in the same tissue increased when the rats inhaled the mixed air pollutants, and was higher than that of the control group. The tendency toward TNF-α mRNA expression significantly increased in 1d and 15d of exposure to mixed air pollutants,
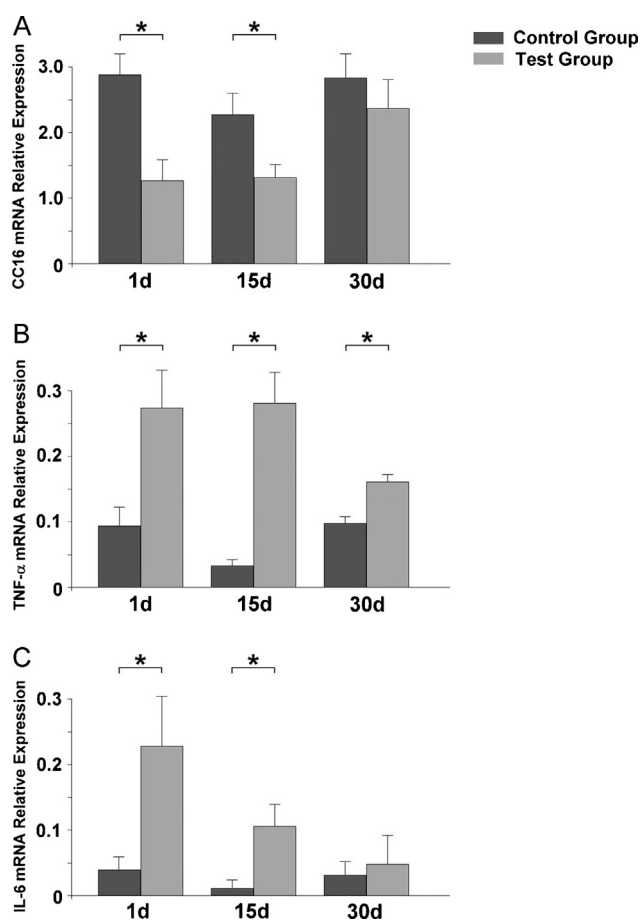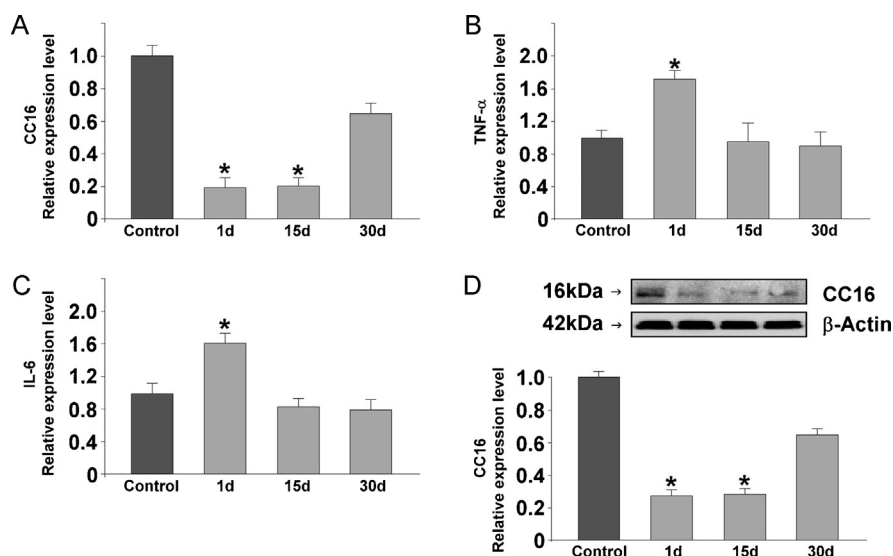

Fig. 3 – The mRNA expression of proinflammatory factors or cytokines in the respiratory tract of rats induced by mixed air pollutants. Relative mRNA levels of proinflammatory factors (A, CC16; B, TNF-α; C, IL-6) as determined by real-time RT-PCR. GAPDH was used as internal control. *$p < 0.05$ when comparing levels of mRNA to control. Data (mean ± s.e.) are representative of the results derived from a minimum of three independent experiments.

and then decreased in 30d, but was still significantly higher than that of the control group (Fig. 3B). Cytokine IL-6 mRNA expression, also increased when the rats inhaled the mixed air pollutants in 1d and 15d, and decreased again by 30d post-exposure (Fig. 3C).

3.2.2. Effects of mixed air pollutants on CC16, TNF-α and IL-6 levels in BALF

In the respiratory tract of treated rats, ELISA and western blot results were consistent with those of mRNA analysis that the protein expression of CC16 in BALF was lower than that of the control group in 1d and 15d treatments but recovered by 30d (Fig. 4A, D). The ELISA results for TNF-α and IL-6 showed that these cytokines at 1d post-treatment were significant higher than those of the control group, and neither 15d nor 30d treatment had statistically significantly effects on cytokine expression (Fig. 4B, C).

Fig. 4 – The protein expression of proinflammatory factors or cytokines in the respiratory tract of rats induced by mixed air pollutants. Relative protein levels of proinflammatory factors as determined by ELISA (A, CC16; B, TNF-α; C, IL-6) and western blot (D, CC16). WB results were normalized to β-actin as a control and densitometric analysis of bands was carried out with the imageJ software. *$p < 0.05$ when comparing levels of protein to control. Data (mean ± s.e.) are representative of the results derived from a minimum of three independent experiments. Effects of mixed air pollutants on CC16, TNF-α and IL-6 levels in the BALF.

4. Discussion

Mixed air pollutants can cause a variety of serious health problems. When exposed to these pollutants, people with heart or lung diseases and older adults are more at risk of hospital and emergency room visits (Granados-Canal et al., 2005; Krewski and Rainham, 2007; Samet and Krewski, 2007; Yang and Yang, 1994). If a person has lung disease, and is exposed to the mixed air pollutants, they may not be able to breathe as deeply or vigorously as normal. Mixed air pollutants can also increase a person's susceptibility to respiratory tract infections. In healthy humans, the respiratory tract including the lung, small bronchi, and pleura are sterile, and without permanent bacterial colonization. However, there are about $1.6 \times 10^6 \, \text{ml}^{-1}$ of *Staphylococcus* and $6.3 \times 10^6 \, \text{ml}^{-1}$ of anaerobic *Lactobacillus* in the nasal mucous membranes (Kang, 1998). The bacterial flora in the oropharynx are generally in balance serving in a commensal relationship with the human host, whereby normal bacterial flora act as an important microbial barrier to defend against other pathogenic bacteria from colonizing the lower respiratory tract and lung (Rowland, 1988; Wilson et al., 1996).

The results presented here demonstrate that the microecosystem of the upper respiratory tract of rats is perturbed by mixed air pollutants, and that the microorganisms incurred quantitative, qualitative or positional changes following exposure to mixed air pollutants. The functions of the normal microorganism biological barrier were compromised, and some microorganisms changed pathologically. The density of anaerobic bacteria increased significantly for 1d exposure to the mixed air pollutants, but decreased in 15d, and then increased again after 30d. This suggested that the

microecology balance was disrupted with early pathologic changes, but with extended exposure time, the host appeared able to compensate. However, as the exposure time increased, the compensatory state was disrupted, causing pathogenic progress.

With exposure to the mixed air pollutants, CC16 mRNA expression initially decreased at 1d and 15d post-exposure, but subsequently rose to a higher level at 30d. The same pattern was also found in CC16 protein expression in BALF. We speculate that the reason for phenomenon in CC16 expression was possible a host compensatory response. It is possible that the mixed air pollutants might interfere with the biologic functions of Clara cells in lung tissue thereby down-regulating CC16 expression in early inflammatory responses. With the progression of acute to chronic inflammation, large amounts of CC16 were secreted into the lung tissue to continue to enhance the CC16 anti-inflammatory effect.

In this study, we observed that the mRNA expression of TNF-α and IL-6 in the lung tissue increased in 1d and 15d and decreased by 30d with exposure to the mixed air pollutants. The same pattern was also found in protein expression levels of TNF-α and IL-6, suggesting that TNF-α and IL-6 may play important roles in mediating the acute pro-inflammatory phase of lung tissue damage.

Additionally, we investigated the relationship with CC16 and cytokines in the inflammatory responses. It has been reported that many cytokines such as TNF-α and IFN-γ could promote the prolongation of the CC16 mRNA half-life and also modulate CC16 mRNA expression at the level of transcription, while inducing IL-2 action on peripheral monocytes and reducing TNF-α synthesis. Thus, we speculate that CC16 likely acts by antagonizing endogenous cytokine and modulates inflammatory cytokine responses. Magdaleno has previously

shown that TNF-α can specifically decrease the function of CC16 gene initiation factor and inhibit CC16 gene transcription. Our study showed that mixed air pollutants decreased the mRNA expression of CC16 in rat lung tissue while inducing the mRNA expression of TNF-α, IL-6. This implied that there may be an interaction between CC16 and inflammatory cytokines. However, further studies will more closely investigate the mechanistic basis for of this interaction.

In conclusion, this study has shown that the equilibrium of the oropharyngeal microecosystem was perturbed by exposure to mixed air pollutants, and that the microorganisms incurred quantitative, qualitative or positional changes following exposure. These changes to the oropharyngeal microecosystem correlated with histologic evidence of injury to the ciliated bronchiolar epithelial cells, interference with CC16 secretion, and of the induction of pro-inflammatory cytokine expression such as TNF-α and IL-6. These changes correlated with injury to the bronchiolar epithelial cells and exacerbation of inflammatory responses, resulting in the progression of respiratory disease.

Conflict of interest statement

Nothing declared.

Acknowledgement

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