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Middle ear inflammation of rat induced by urban particles

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ABSTRACT

Objective: The aim of this study was to evaluate the histologic change of middle ear mucosa and the expression levels of epithelial sodium channel (*ENaC*) subunits and mucin production genes, after the injection of urban particulate matter (UPM) into the middle ear cavity of rats.
Methods: Fifty pathogen-free, male Sprague Dawley rats were assigned to the study. Transtympanic injection of UPM solution (300 μg/ml, 50 μl) was made into the middle ear cavity of rats. Rats were sacrificed at day 1 (group1); day 3 (group2); day 5 (group3); and day 14 (group4) after the procedure. The expression levels of *ENaC* subunits (α, β and γ) and mucin producing genes (*MUC5AC* and *MUC5B*) were analyzed using semi-quantitative real-time reverse transcriptase–polymerase chain reaction. Thickness of middle ear mucosa was measured and analyzed.
Result: After transtympanic injection, the thickness of middle ear mucosa increased significantly on day 1, 3 and 5 ($p < 0.05$) and was normalized on day 14, compared to the control group. Inflammatory changes observed in the middle ear mucosa were subepithelial widening, inflammatory cell infiltration and vascular space widening on day 1, 3 and 5. These changes had reverted to normal on day 14. The level of *ENaC-*α expression decreased 0.60 fold on day 1 ($p < 0.05$), but was normalized thereafter. The level of *ENaC-*β and γ decreased 0.39 and 0.27 fold, respectively, on day 1, was normalized on days 3 and 5, and increased 2.30 and 2.47 fold on day 14, respectively ($p < 0.05$). The level of *MUC5AC* expression increased 1.97-fold on day 1 ($p < 0.05$) and 2.58-fold on day 5 ($p < 0.05$), but was normalized on day 14. The level of *MUC5B* expression increased 5.4-fold on day 1, 3.14-fold on day 3, 3.85-fold on day 5, and 2.46-fold on day 14, respectively ($p < 0.05$).
Conclusion: Transtympanic injection of UPM solution into the middle ear cavity of rat induced a characteristic inflammatory response and altered gene expression related with inflammation and mucin production. These findings provide a useful clue for the understanding of how air pollutants, particularly UPM, contribute to the development of otitis media.

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1. Introduction

Otitis media (OM) is the most common disease of childhood, second only to upper respiratory infection [1]. It is a significant disease because OM can lead to economic burden for the medications and surgical procedures as well as a conductive hearing loss [2].

The cause of OM is thought to be multifactorial, including a upper respiratory tract infection, allergy, biofilm formation, congenital anomaly, bacterial infection, and environmental factors, such as smoking, and air pollution [3,4].

Ambient air pollutants can induce an inflammatory response in respiratory epithelium and make the respiratory system more susceptible to infection [5,6]. Environmental tobacco smoke is a strong risk factor for OM [7], likely due to alteration of immune defenses and increased susceptibility following inflammation of the respiratory epithelium [8,9]. Inhaled pollutants have induced inflammatory responses in airways and caused asthma in a mouse model [10].

Urban particulate matters (UPM) are fine particles in the ambient air collected in urban area. Recent study showed that traffic-related air pollution in urban area associated with OM [11]. In our previous study, we have reported that various air pollutants including diesel exhaust particle (DEP), UPM, and acrolein, which is one of the hazardous air pollutant in both the environment and tobacco smoke, can induce the gene expression relate to inflammatory response in human middle ear epithelial

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cells (HMEECs) [12–15]. However, there is little data on the effect of air pollutants on the development of OM in animal models.

Middle ear is an air filled cavity lined with respiratory epithelia and fluid clearance is very important for transmission of sound. Epithelial sodium channel (*ENaC*) is essential for maintaining a fluid-free airway lumen by controlling the peri-mucosal fluid [16]. The expression of *ENaC* is known to be altered on Eustachian tube obliteration of rats [17]. Mucin production is closely related with middle ear inflammation and *MUC5AC* and *MUC5B* are known to be the major mucins accumulated in the middle ear effusion in OM [18,19].

We hypothesized that UPM injection into the middle ear cavity would induce inflammatory response in the middle ear mucosa. Accordingly, in this study, we investigated the histological change of the middle ear mucosa and the expression levels of *ENaC* subunits and mucin production genes after the injection of UPM into the middle ear cavity of rats.

2. Materials and methods

2.1. Preparation of UPM

UPM (SRM 1648; http://www-s.nist.gov/srmors/view_detail.cfm?srm=1648) was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, USA). 300 μg/ml UPM was suspended in phosphate buffered saline (PBS, pH7.4). To minimize aggregation, particle suspensions were sonicated for 15 min and vortexed prior to use.

2.2. Animals and experimental design

Fifty pathogen-free, male Sprague Dawley rats weighing 150–200 g (Orient bio, Gyeonggi, Korea) were assigned to the study. The Institutional Animal Care and Use Committee approved the protocols for this study, and the animals were cared for in accordance with Dongguk University Ilsan Hospital. The rats were assigned randomly to the four groups that received a unilateral tympanic membrane UPM injection (groups for day 1, 3, 5 and 14) and the control group that received a PBS injection (vehicle control group). In this experiment, 50 μl of UPM (300 μg/ml) dissolved in PBS was injected using a 27-ga spinal needle. The rats underwent the UPM injection and then animals were sacrificed on day 1 (group1), day 3 (group2), day 5 (group3), and day 14 (group4) post-procedure. The rats were anesthetized with IP ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg) injection.

2.3. Real-time reverse transcription polymerase chain reaction (RT-PCR)

Five rats from each experimental group were sacrificed on days 1, 3, 5 and 14. After acquiring middle ear bulla, tympanic membrane and surrounding bone was removed. The middle ear mucosa was exposed and dissected with pick. Whole middle ear mucosa covering bulla, promontory, and Eustachian tube area was acquired [17].

The total RNA was then extracted using an RNeasy® Mini kit (QIAGEN, Hilden, Germany). The SuperScript II reverse transcriptase was used to synthesize cDNA (Invitrogen). After reverse transcription, semi-quantitative real-time RT-PCR was performed using a LightCycler 480 Real-time RT-PCR System (Roche, Basel, Switzerland) with the following amplification conditions: denaturation at 95 °C for 5 min; followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; then extension at 72 °C for 5 min. Each reaction mixture contained 10 μl LightCycler 480 SYBR Green I Master (Roche), 0.5 μl of 6 pmol each of sense and antisense primers, and 0.4 μl of cDNA in a final volume of 20 μl. Amounts of target mRNA were normalized to endogenous *GAPDH* expression, and target mRNA expressions in the experimental groups were calculated, relative to the control group. The primers for *ENaC-α*, *ENaC-β*, *ENaC-γ*, *MUC5AC*, *MUC5B*, and glyceraldehyde-3-phophate dehydrogenase (GAPDH) are shown in Table 1.

2.4. Histological analysis

Five rats from each of the experimental groups (groups 1, 2, 3, and 4) and five rats from control group were sacrificed and then isolated bullae (including both middle and inner ear) in brain of rats. Each bullae were fixed in 4% paraformaldehyde for 48 h and decalcified with Calci-Clear Rapid (National Diagnostics, Atlanta, GA, USA) for 24 h at room temperature. The specimens were dehydrated and embedded in paraffin. Each histological section (5 μm thickness) was stained with hematoxylin–eosin. From each block, 10 sections that contained the central portion were selected and stained. The specimens was examined under a binocular microscope (Leica DMI 6000B; Leica Microsystems, Wetzlar, Germany) equipped with a camera (Leica DFC480; Leica Microsystems, Herburgg, Switzerland). Thickness of middle ear mucosa was measured in pixels using a microscopic computerized image analysis program (Leica Q win V3, Leica Microsystems Ltd., Bellinzona, Switzerland).

2.5. Statistical analysis

All data were expressed as mean ± standard deviation. Student's *t* test was used to determine statistically significant differences between control and experimental groups. $p < 0.05$ for the null hypothesis was accepted as a statistically significant difference. Statistical analyses were performed using SPSS version 11(SPSS Inc., Chicago, Il, USA).

3. Results

3.1. Histological analysis of middle ear mucosa

Normal middle ear mucosa was composed of single layered epithelium and subepithelial tissue attached to bony bulla (Fig. 1A). At day 1, sub-epithelial space in middle ear became widened (Fig. 1 B) and the thickness of middle ear mucosa increased and subepithelial space was widened even more (Fig. 1C and D). At day 14, the thickness of middle ear mucosa was normalized and histological feature were comparable with vehicle control (Fig. 1E).

Epithelium of middle ear was thickened and cilia and infiltration of inflammatory cells became visible at day 1 after

Table 1
Primer sequences and conditions used for the real-time RT-PCR.

Gene	Accession no.	Primer sequences	Product size (bp)
MUC5AC	XM_003749022	5′-ACTATGAGGTGCGACTGCTT-3′ 5′-CTTGTGGGATGTCACAGGAGT-3′	80
MUC5B	XM_001063609	5′-GGCCTCTGGCAAGAAGATGT-3′ 5′-GTTCTCTGCCCGACACTCAA-3′	58
ENaC-α	NM_031548	5′-ACTGTCTGCACCCTTAATCCTT-3′ 5′-GTGATGCGGTCCAGCTCT-3′	71
ENaC-β	NM_012648	5′-GGCATGACAGAGAAGGCACT-3′ 5′-GACCAATGTCCAGGATCAACTT-3′	76
ENaC-γ	NM_017046	5′-AGAAAATGCCACCATCCTCA-3′ 5′-TCTTCGTTTATGTATAAGATGA-CTTGC-3′	78
GAPDH	NM_017008	5′-TGGGAAGCTGGTCATCAAC-3′ 5′-GCATCACCCCATTTGATGTT-3′	78

MUC: mucin; ENaC: epithelial sodium channel; GAPDH: glyceraldehydes-3-phosphate dehydrogenase.

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