The potential cytotoxic effects of urban particle matter on olfaction*

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Abstract

Background: Urban particulate matter (UPM) in ambient air is implicated in a variety of human health issues worldwide, however, few studies exist on the effect of UPM on the olfactory system. This study aimed to identify the factors affecting the destruction of the olfactory system in a mouse model following UPM exposure.

Methods: Mice were divided into: control and four UPM-exposed groups (200 μg UPM at 1 and 2 weeks, and 400 μg UPM at 1 and 2 weeks [standard reference material 1649b; average particle diameter 10.5 μm]). The olfactory neuroepithelium was harvested for histologic examination, gene ontology, quantitative real-time polymerase chain reaction, and western blotting.

Results: Compared to the control group, olfactory marker protein, Olfr1507, ADCY3, and GNAL mRNA levels were lower, and S-100, CNPase, NGFRAP1, BDNF, and TACR3 mRNA levels were higher in the olfactory neuroepithelium of the UPM groups. Moderately positive correlation was present between the 1- and 2-week groups. After analyzing the 200 and 400 UPM groups separately, the strength of the association between the 200 UPM 1- and 2-week groups was moderately positive. No differences was present in the neuroepithelial inflammatory marker levels between the UPM and control groups.

Conclusions: UPM could have cytotoxic effects on the olfactory epithelium. The exposure time and particular concentration of UPM exposure could affect the degree of destruction of the olfactory neuroepithelium. The olfactory regeneration mechanism could be related to the neurotrophic factors, olfactory ensheathing cell stimulation, and trigeminal nerve support.

Key words: urban particulate matter, olfaction, olfactory dysfunction, time to exposure

Introduction

Many studies have disclosed causes of olfactory disturbances. Conductive causes include diseases of the nasal and paranasal sinuses. Sensory-neural causes include upper respiratory tract infection (URI), traumatic head injury, neurodegenerative disorders, and toxins [(1)]. Exposure to ambient air pollution, especially to urban particulate matter (UPM), is one source of direct toxin to the olfactory epithelium.

Numerous epidemiological studies indicate that long- and short-term exposure to UPM is associated with adverse health effects on the human respiratory system [(2)]. For example, exposure to UPM resulted in alterations in the levels of the cytoplasmic and mitochondria-targeted transcripts associated with apoptosis organization in the human olfactory mucosa [(3)]. Considering the direct exposure of olfactory receptors to the external environment, olfaction is particularly vulnerable to damage from toxicants [(4)]. Some toxicants have the potential to damage not only the olfactory receptor cells, but also the central nervous system structures by entering the brain through the olfactory mucosa [(5)]. Furthermore, damage to the olfactory neuroepithelium can reportedly occur directly as an acute or chronic injury, or indirectly via the induction of inflammatory processes [(6)]. Therefore, we aimed to investigate the cytotoxic effects of UPM on the olfactory epithelium, based on accurate

scientific experiments and the mechanism of corresponding changes in cytokine levels. We also sought to identify the factors affecting the destruction of the olfactory system following exposure to UPM, based on scientific evidence. We used valuable olfactory markers and neurotrophic factors to prove the effect of UPM. These markers are explained in detail in the methods and discussion sections.

Materials and methods

Real-time polymerase chain reaction

We evaluated OMP, Olfr1507, ADCY3, and GNAL mRNA levels in the olfactory neuroepithelium, using real-time polymerase chain reaction (RT-PCR). Moreover, we analyzed S100 and CNPase mRNA levels in the olfactory neuroepithelium to demonstrate the change of olfactory ensheathing cells. We evaluated the NGFRAP1 and BDNF mRNA levels in the olfactory neuroepithelium to demonstrate the change in the neurotrophic effect in the olfactory neuroepithelium. We analyzed TACR mRNA levels in the olfactory neuroepithelium to demonstrate the change of trigeminal nerve support to olfactory neuroepithlium. Finally, we analyzed the levels of the inflammatory markers; TNF-α, IFN-γ, IL-1, IL-5, IL-6, IL-10, IL-12, and IL-17 in the serum and nasal tissue.

Subjects

Thirty-six healthy female C57BL6 mice (aged 9–10 weeks and weighing 18–20 g each) were allocated to the following five groups based on their exposure to different UPM levels: control, n=12; 200 UPM 1 week, n=6; 200 UPM 2 weeks, n=6; 400 UPM 1 week, n=6; and 400 UPM 2 weeks, n=6. All the animals received care according to the Institutional Animal Care and Use Committee guidelines. Mice in the UPM groups received UPM (200 μg/20 μl; 10 μl of 200 UPM in each nostril, 400 μg/20μl; 10 μl of 400 UPM in each nostril) intra-nasally for 1 or 2 weeks. Mice in the UPM group received UPM intra-nasally once per day for 1 week in the "1-week groups" and 2 weeks in the "2-weeks groups." They were sacrificed the day after the administration period ended. Mice in the control group received saline intra-nasally once per day for 1 or 2 weeks. Mice were allocated to each group using randomisation lists generated by an online randomiser tool (www.randomizer.org) provided by the coordinating center. The animal experiments of this study were approved by the Institutional Animal Care and Use Committee, Uijeongbu St. Mary's Hospital, The Catholic University of Korea (No. UJA2017-10A).

Reagents

UPM (standard reference material 1648a; average particle diameter 10.5 μm, Sigma Aldrich, USA) was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). It consists of polycyclic aromatic hydrocarbons, polychlorinated biphenyl congeners, chlorinated pesticides, and other atmospheric particulate materials.

Identification and harvesting of the whole olfactory neuroepithelium

The mice were sacrificed following 1 or 2 weeks UPM administration. We randomly took samples from the whole head for immunohistochemistry, ethmoturbinate, PCR, and western blot. All the dissection procedures were performed under a microscope. The posterior half of the nasal cavity contains convoluted structures formed by a bone core called the ethmoturbinate and is lined by olfactory epithelium, which contains olfactory receptor neurons. The entire ethmoturbinate was harvested using micro scissors, and the olfactory neuroepithelium was used for western blotting and RT-PCR.

Tissue preparation

Immunohistochemistry was performed as previously described [10]. Briefly, the heads of the mice, including the olfactory neuroepithelium and the olfactory bulb, were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich, Chicago, IL, USA), decalcified using 20% ethylenediaminetetraacetic acid (pH 7.4) at room temperature for 4 weeks, and then embedded in paraffin.

Hematoxylin and eosin staining

The samples were sectioned at 4 μm and stained with hematoxylin and eosin. Histological changes in tissues were evaluated microscopically, using an Olympus BX50 microscope (Olympus, Japan).

Immunohistochemistry

Immunohistochemistry was performed using a commercial avidin-biotin complex kit (Vectastain® Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, deparaffinized sections were subjected to antigen retrieval in a microwave for 15 min with 10 mM sodium citrate buffer (pH 6.0), and then treated with 3% hydrogen peroxide in methyl alcohol for 15 min to block endogenous peroxidase activity. The samples were incubated with primary antibody rat monoclonal anti-Substance P (1:25, Santa Cruz, CA, USA) and rabbit polyclonal TACR3 (1:200, Bioss, CITY, USA) overnight at 4°C. Substance P is released from the fifth cranial nerve, the trigeminal nerve, which is part of trigeminal system [7]. After washing three times with TBST (Tris-buffered saline, 0.1% Tween 20) buffer, sections were sequentially reacted with the appropriate biotinylated secondary antibody followed by an avidin-biotin-peroxidase complex. The peroxidase reaction was conducted using a 3-3'-diaminobenzidine substrate kit (Vector Laboratories), followed by counterstaining with hematoxylin, before mounting. The staining results were captured with a camera attached to an Olympus BX50 microscope.

Total RNA extraction

Total RNA was isolated using the TRIzol reagent (Invitrogen, USA). RNA had an A260/A280 ratio >1.7, which was quantified using a spectrophotometer (Nanodrop ONE C; Thermo Fisher Scientific, Inc., USA).

RT-PCR

RNA was obtained using TRIzol reagent, and cDNA was synthesized using a cDNA synthesis kit (Promega, CITY, USA) according to the manufacturer's instructions and included a final DNase I (Thermo Fisher) step. RT-PCR was performed using the GoTaq® qPCR master mix (Promega) on the CFX96 Real-Time System (Bio-Rad). The primer sequences were as follows: olfactory marker protein (OMP), F: 5'-CGACCTCACCAACCTCATGA-3', R: 5'-CATGACCTTGCGGATCTTGG-3'; GNAL, F: 5'-GACTACACACCCACAGACCA-3', R: 5'-GCCACGTAAATGATCGCAGT-3'; Olfr1507, F: 5'-GAAAGCCTTGTCCACCTGTG-3', R: 5'- GGGTTCAGCAGAGGGGTTAT-3'; ADCY3, F: 5'-GGACACGCTCACAAACATC-3', R: 5'-GCCACATTGACCGTATTGC -3'; S100, F: 5'-AAAGTGAT GGAGACGCTGGA-3', R: 5'-CTTTGCTGTGCCTCCTCTTG -3'; CNPase, F: 5'-GCTGCAGTTCCCTTTCCTTC -3', R: 5'-CATCAGCGGACACCATCTTG -3'; NGFRAP1 F: 5'-CATTCCCA ACAGGCAGATG -3', R: 5'-GGCATAAGGCAGAATTCATC -3'; BDNF F: 5'-TTGTTTTGTGCCGTTTACCA-3', R: 5'-GGTAAGAGAGCCAGCCACTG -3'; IL10 F: 5'-GGACAACATACTGCTAACCGAC-3', R: 5'-TGGATCATTTCCGATAAGGCTTG-3'; TACR3 F: 5'-CCAACTACTGCCGCTTCCA-3', R: 5'-GAAATGTTGCTTGGGACCTTCT-3'; IFN-gamma: 5'-CTTCTTCAGCAACAGCAAGG-3', R: 5'-TGAGCTCATTGAATGCTTGG-3'; IL-17 F: 5'-GCTCCAGAAGGCCCTCAGA-3', R: 5'-CTTTCCCTCCGCATTGACA-3'; IL-1 F: 5'-TCTGCCATTGACCATCTC-3', R: 5'-GAATCTTCCCGTTGCTTG-3'; IL-6 F: 5'-ATCAGGAAATTTGCCTATTGAAA-3', R: 5'-CCAGGTAGCTATGGTACTCCAGA-3'; TNF-alpha F: 5'- GACGTGGAACTGGCAGAAGAG-3', R: 5'-GCCACAAGCAGGAATGAGAAG-3'; IL-5 F: 5'-GGCTGGCCTCAAACTGGTAA-3', R: 5'-CCCTGATGCAACGAAGAGGA-3'. Finally, GAPDH, F:5'-TGTTGTGTCCGTCGTGGATCTGA-3', and R:5'-TTGCTGTTGAAGTCGCAGGAG-3' were used as a reference pair to calculate the fold change in target gene expression. Cycle threshold values were estimated for the target genes, and GAPDH was used as a housekeeping gene.

TACR3 belongs to a family of genes that function as receptors for tachykinins. The TACR3 mRNA level evaluation could indicate trigeminal nerve stimulation or destruction for the maintenance of the olfactory neuroepithelium. The olfactory marker protein (OMP) is a protein involved in signal transduction. Olfr1507 is one of the olfactory receptor proteins to whihc an antibody is easily obtained. Protein d had previsoulsy shown good presentation in the olfactory mucosa in our studies [9-11]. In those studies, one consistent observation was that all cultured OECs, regardless of the olfactory source, S100 and CNPase markers can be used to reliably localize mucosal and bulbar OECs in animal models [12].

Western blotting

Samples obtained from the experimental mice were dipped in liquid nitrogen and stored at -70°C until required. Samples were homogenized in 500 µL of Pro-Prep protein extraction solution (Intron Biotechnology, Korea) for 1 min. The homogenate was lysed by incubation on ice for 10 min, and then centrifuged at 14,000 rpm for 20 min at 4°C. Protein concentration was measured using a bicinchoninic acid assay. For each sample, 30 µg of protein was mixed with a sample buffer and heated at 100 °C for 10 min. The proteins were seperated on 8-15% SDS-PAGE gels, and then transferred to a nitrocellulose membrane (Amersham, Sweden). Thereafter, the membranes were rinsed in TBS with 0.1% Tween-20, incubated for 1 h in TBS containing 5% skimmed milk, and then incubated with the following primary antibodies: rabbit anti-OMP (Abcam, UK), mouse anti-S100 (Thermo Fisher Scientific, USA), rabbit anti-GAPDH (Cell Signaling, USA), rabbit anti-olfr1507 (Thermo Fisher Scientific, Inc., USA), and rabbit anti-GNAL (Novus, USA). After incubation with the primary antibodies at the proper dilution, the blots were incubated with the corresponding secondary antibody conjugated with horseradish peroxidase (Bio-Rad, USA). Immunoreactivity was visualised using the western blotting ECL reagent (Santa Cruz Biotechnology, USA), and immunoreactive band signal intensities were quantified using the CS Analyzer 3.0 (ATTO Corporation, Japan) and normalized to the corresponding signal for GAPDH.

Data analysis

The standard concentration-specific reaction was calculated using the MasterPlex QT 2010 software (MiraiBio, Hitachi, USA), which automatically selects the highest R square (maximum = 1.0) curve for each calculation method to obtain the best fit. Each sample result was calculated based on this standard curve to reflect the dilution ratio. The concentration value of the sample was calculated by accounting for its data distribution as much as possible when adjusting for the standard curve. The Pearson correlation coefficient between the UPM dose and exposure time of the change in the olfactory system-related mRNA expression was calculated using IBM Statistical Package of the Social Sciences Statistics for Windows, version 21 (IBM Corp., Armonk, NY, USA). Correlation coefficient values >0.7 were defined as strong, 0.4 to 0.7 as moderate, 0.2 to 0.4 as weak, and 0 to 0.2 as no correlation. Statistical significance was set at $p < 0.05$.

Results

Hematoxylin and eosin staining

Histological changes in the olfactory mucosa were evaluated using bright-field microscopy. After 2 weeks, the olfactory mucosa in the control group presented an ordered epithelium with

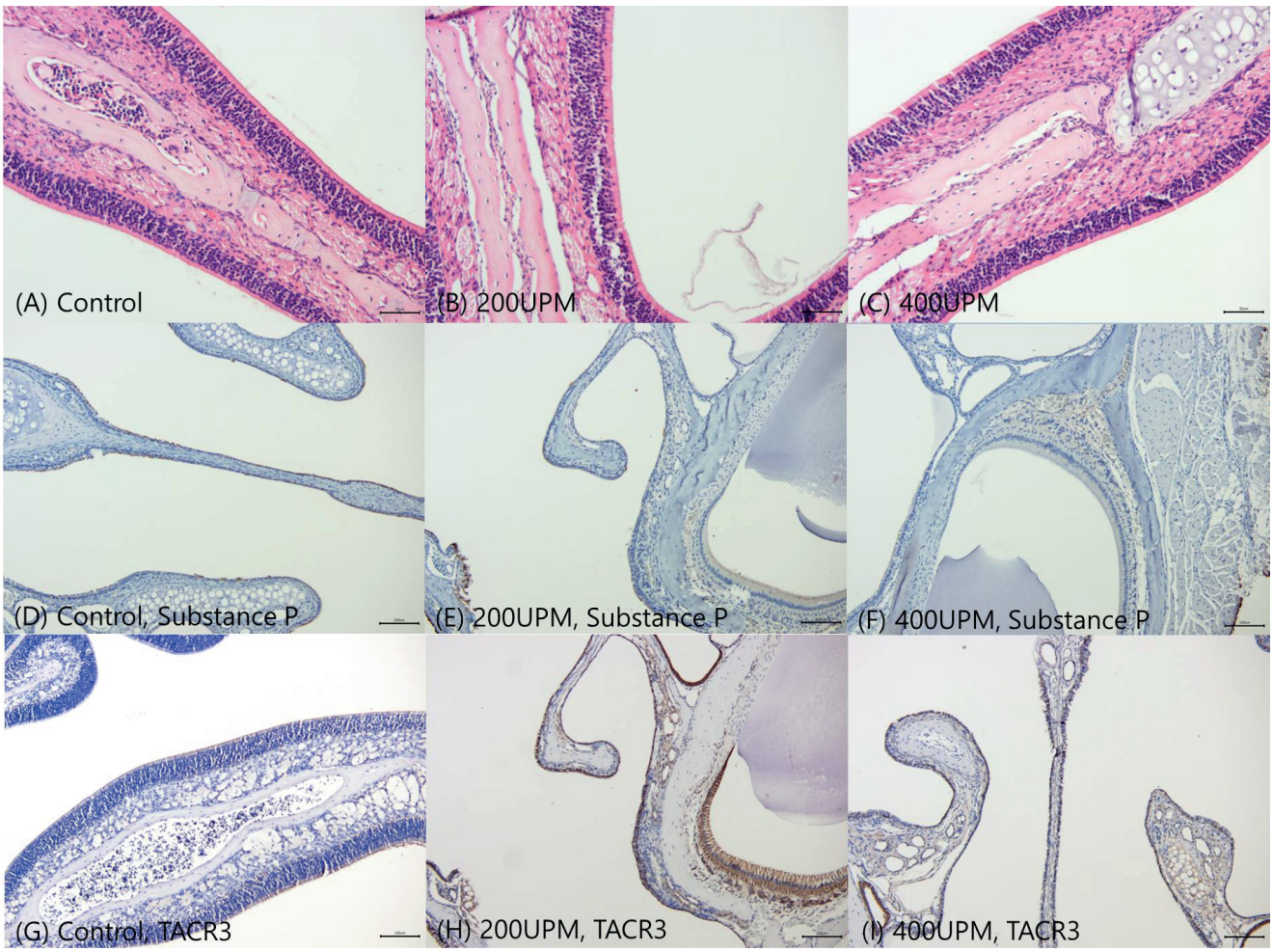

Figure 1. (A) The olfactory mucosa in the control group had an ordered epithelium with a straight basement membrane zone. (B) and (C) In the 200 and 400 UPM group, the olfactory epithelium and Bowman's gland were hypoplastic and poorly organized with mitotic figures, and the lamina propria expanded due to edema. Substance P (D, E, F) and TACR3 (G, H, I) demonstrates differences in the extent of trigeminal fibers across the three defined groups, control, 200 UPM and 400 UPM groups. In E, F, H, I cases, the labeling is concentrated at the basal lamina. UPM: urban particulate matter; E, H: 200UPM; F,I:400UPM.

a straight basement membrane zone, and mitotic figures were not observed. In the 200 and 400 UPM groups, the olfactory epithelium and Bowman's gland were poorly organized, and the lamina propria expanded owing to edema (Figure 1 A-C).

Immunolocalization of substance P and TACR3

Substance P and TACR3 expression were detected in the olfactory mucosa and basal lamina. Based on substance P and TACR3 staining in the control, 200 UPM, and 400 UPM groups, the labeling was concentrated in the basal lamina, while the labeling density was increased in the 200 UPM group and greatly increased in the 400 UPM group (Figure 1 D-I).

RT-PCR and western blot analyses

To identify the factors affecting the olfactory epithelial cells exposed to UPM, we compared the changes in the olfactory system-related mRNA expression based on the concentration of UPM and the exposure time. OMP, Olfr1507, ADCY3, and GNAL mRNA levels in the olfactory neuroepithelium were evaluated using RT-PCR and were reduced in the 200 UPM and 400 UPM groups in comparison to the control groups at both 1 and 2 weeks. OMP, Olfr1507, ADCY3, and GNAL mRNA levels in the olfactory neuroepithelium were more reduced at the 2 week timepoint than after 1 week. OMP and GNAL mRNA levels were significantly reduced in the 200 UPM group compared to the control group at 2 weeks (p-values: OMP, control vs. 200 UPM in 2 weeks, 0.028; Olfr1507 control vs. 200 UPM in 2 weeks, 0.062; ADCY3 control vs. 200 UPM in 2 weeks, 0.106; GNAL control vs. 200 UPM in 2 weeks, 0.033) (Figure 2A).

S100, CNPase, NGFRAP1, BDNF, and TACR3 mRNA levels in the olfactory neuroepithelium were also evaluated using RT-PCR and were found to be higher in the 200 UPM and 400 UPM

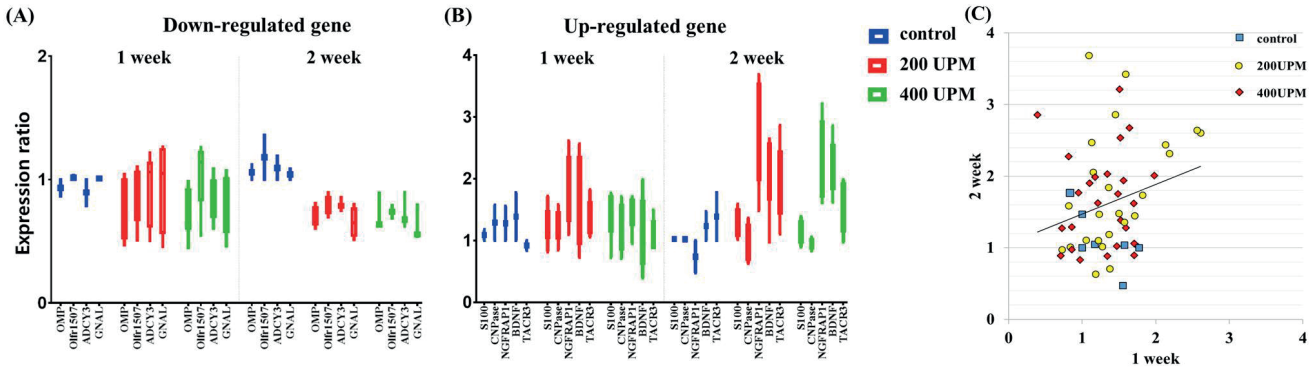

Figure 2. (A) OMP, Olfr1507, ADCY3, and GNAL mRNA levels in the olfactory neuroepithelium were evaluated using RT-PCR and were found decreased in the 200 UPM and 400 UPM group compared to the control group in both 1 and 2 weeks. (B) S100, CNPase, NGFRAP1, BDNF and TACR3 mRNA levels in the olfactory neuroepithelium were found to be higher in the 200 UPM and 400 UPM groups than the control groups. (C) OMP, Olfr1507, ADCY3, GNAL, S100, CNPase, statistical analysis. Time (1 and 2 weeks) data showed that the strength of association between the variables positively correlated (r = 0.468812), and that the correlation coefficient is very highly significantly different from zero (p = 0.002). RT-PCR: quantitative real-time polymerase chain reaction, OMP: olfactory marker protein, Olfr1507: olfactory receptor 1507, UPM: urban particulate matter.

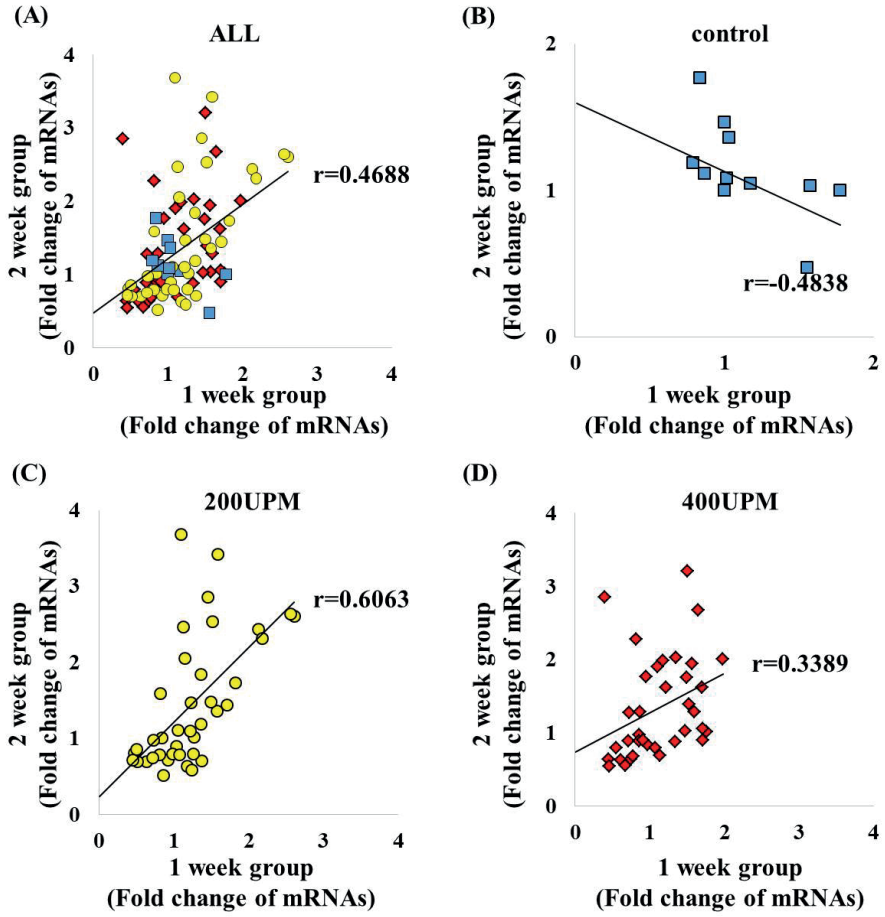

Figure 3. The relationship of changes in olfaction-associated mRNAs (OMP, Olfr1507, ADCY3, GNAL, S100, CNPase, NGFRAP1, BDNF and TACR3) expression levels within the control (blue box), 200 UPM (yellow circle) and 400 UPM (red rhombus) groups for two time periods (1 and 2 weeks) was compared by statistical analysis. Time (1 and 2 weeks) data demonstrated that the strength of association between the variables was negatively correlated (r = 0.4688). The strength of association between the variables was highly positively correlated (r = 0.606302) in the 200 UPM groups, and the correlation coefficient was very highly significantly different from zero (<0.0001). RT-PCR: quantitative real-time polymerase chain reaction, OMP: olfactory marker protein, Olfr1507: olfactory receptor 1507, UPM: urban particulate matter.

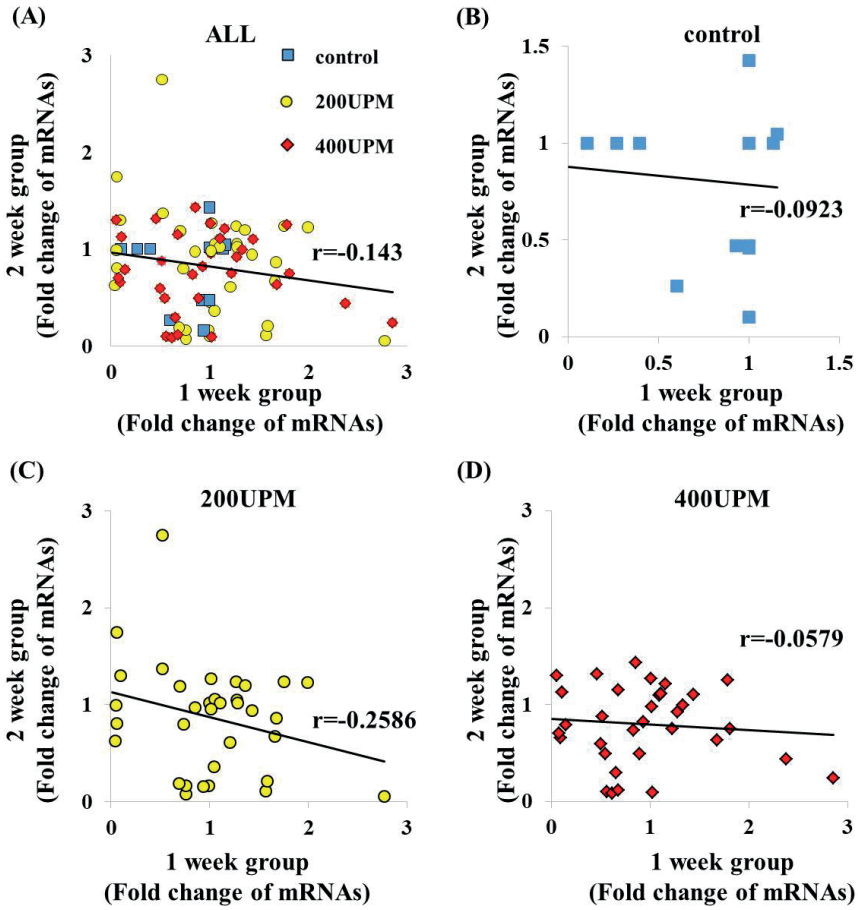

Figure 4. The relationship of the changes in the inflammation-associated mRNAs (TNF-α, IFN-γ, IL-1, IL-5, IL-6, IL-10 and IL-17) expression levels within the control (blue box), 200 UPM (yellow circle) and 400 UPM (red rhombus) groups for two time periods (weeks 1 and 2) was compared using statistical analysis. Time (1 and 2 weeks) data showed that the strength of association between the variables was negatively correlation (r = -0.143). TNF-α: tumor necrosis factor alpha, IFN-γ: interferon-gamma, IL: interleukin, UPM: urban particulate matter.

groups than in the control groups. NGFRAP1 mRNA levels were significantly higher in the 200 UPM group than in the control group at 2 weeks (p-values: S100 control vs. 200 UPM in 2 weeks, 0.4; NGFRAP1 control vs. 200 UPM in 2 weeks, 0.033; BDNF control vs. 200 UPM at 2 weeks, 0.218; TACR3 control vs. 200 UPM at 2 weeks, 0.809) (Figure 2B).

Statistical evaluation of time with UPM exposure and density

We analyzed the correlation between the UPM concentration and exposure time with the changes in olfactory-related mRNA expression. OMP, Olfr1507, ADCY3, GNAL, S100, CNPase, NGFRAP1, BDNF, and TACR3 mRNA levels in the olfactory neuroepithelium were compared. Time (1 and 2 weeks) data demonstrated that the strength of the association between the variables showed a moderately positive correlation (r = 0.468812), which was highly significant (p = 0.002, Figure 2C). We separated the control, 200 UPM, and 400 UPM groups, and then analysed the relationship between the 1- and 2-week groups. We found a significant positive correlation between the variables in the 200 UPM groups (r = 0.606302; p <0.0001) (Figure 3). The correlation score between the 200 and 400 UPM groups was 0.426237 in S100, CNPase, NGFRAP1, BDNF, and TACR3. The correlation score between 200 and 400 UPM was -0.32512 in OMP, Olfr1507, ADCY3, and GNAL in the 2 weeks group. However, the correlation between the two doses (200, 400UPM) showed no significant difference in the analysis of the olfactory and OEC markers.

We also analysed the correlation between the UPM concentration and exposure time with the changes in the inflammatory cytokine-related mRNA expression. Tumor necrosis factor alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-1, IL-5, IL-6, IL-10, and IL-17 mRNA levels in the olfactory neuroepithelium were analysed. Time (1 and 2 weeks) data showed no correlation between the variables (r=-0.143). We separated the control, 200 UPM and 400 UPM groups, and then analyzed the relationship between the 1-and 2-week groups. There was no correlation between the variables in the 200 UPM (-0.2586) and 400 UPM (r=-0.0579) groups (Figure 4).

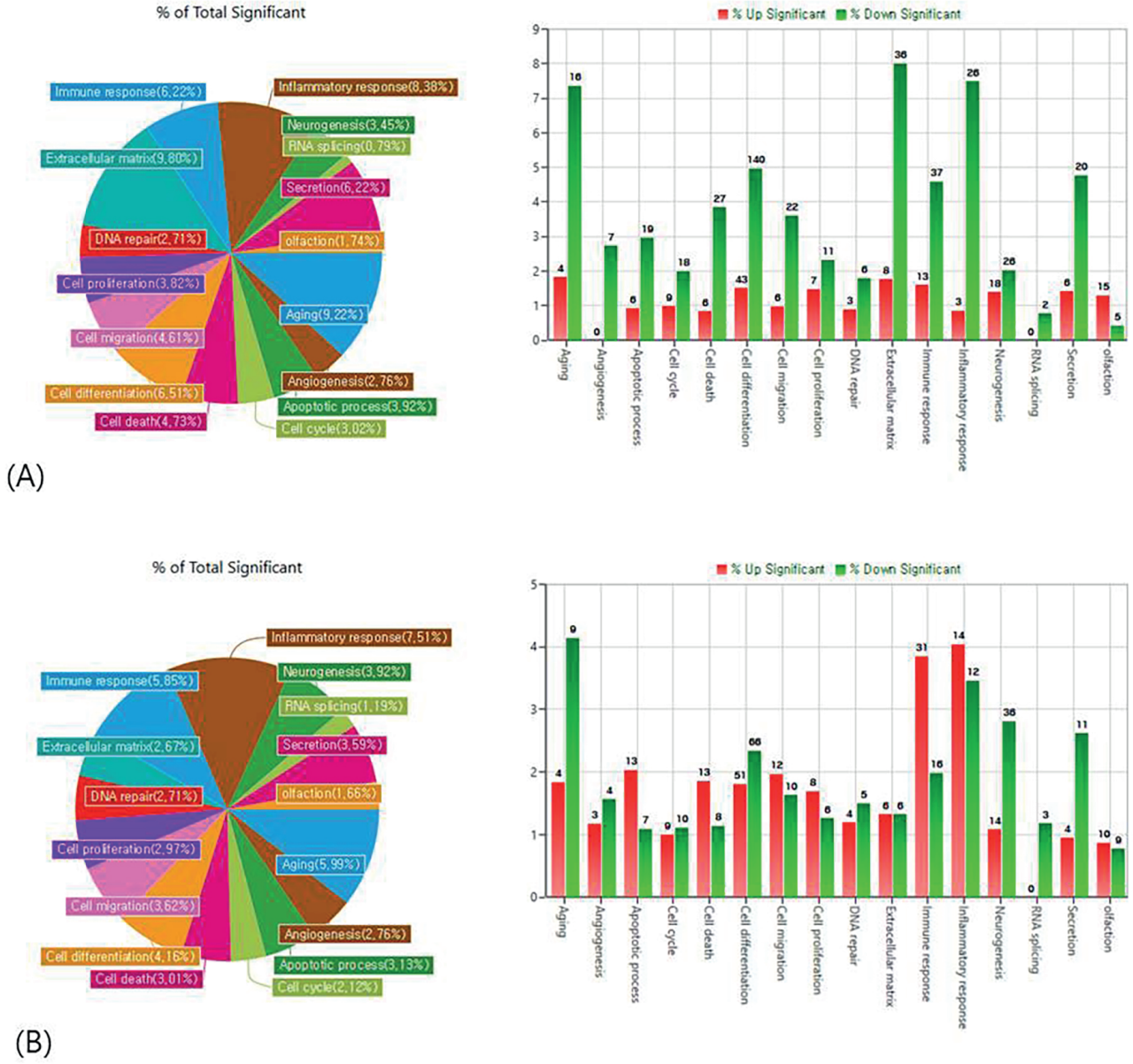

Figure 5. Pie charts and bar graphs of differentially expressed mRNAs and gene ontology analysis results of differentially expressed mRNAs in (A) nasal tissue in the 200 UPM 2-week group and (B) the nasal tissue in the 400-UPM 2-week group. Red: expression level of y-value is higher than the expression level of x-value; Green: expression level of y-value is lower than the expression level of x-value. The genes rleated to the inflammatory response, immune system, and olfaction were altered in the nasal neuroepithelium in the filter gene category plot (fold change, 1.5; log2 normalized read counts ≥ 4 were selected). UPM: urban particulate matter.

Gene ontology (GO)

Scatter plots of the differentially expressed mRNAs and the results of the GO analysis of the differentially expressed mRNAs are shown in Figure 5. We focused especially on neurogenesis, inflammatory response, immune system, and olfaction (fold change of 2, and log2 normalized read count of 4 were used to minimize false counts). Compared to the control groups, mRNAs were upregulated and downregulated in the 400 UPM (4,156 and 4,005, respectively) and 200 UPM (4,322 and 4,271, respectively) 2-week nasal neuroepithelium tissue.

Gene expression related to inflammatory response and immune system was more increased in the 400 UPM than in the 200 UPM nasal neuroepithelium of the filter gene category plot.

Western blot analysis

Western blot analysis was performed to assess the OMP,

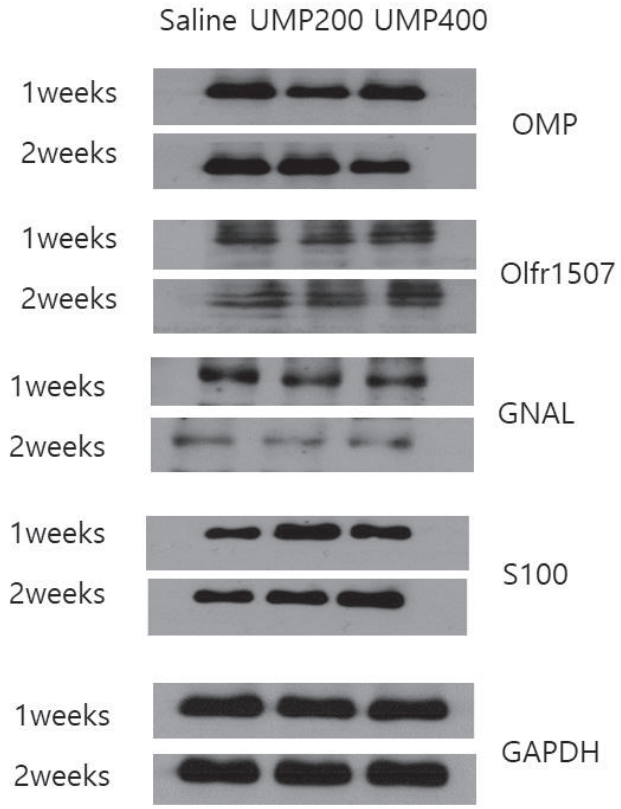

Figure 6. Western blot analysis was performed to assess OMP, Olfr1507, GNAL and S100, protein expression in the 200 and 400 UPM group compared with that in the control group, at 1and 2 weeks. OMP: olfactory marker protein, Olfr1507: olfactory receptor 1507, UPM: urban particulate matter. *400UPM: Mice in 400UPM groups were administered UPM (standard reference material 1649b; average particle diameter 10.5 μm) with 400μg/20μl.

Olfr1507, GNAL and S100, protein expression in the 200 and 400 UPM group compared with that in the control group, at 1and 2 weeks. OMP, Olfr1507and GNAL induction were negatively affected following UPM exposure at 2 weeks. Furthermore, S100 expression increased during 2-weeks (Figure 6).

Discussion

In several previous studies, UPM reduced the cell viability in a dose-dependent manner and increased inflammatory cytokine expression at both the mRNA and protein levels, in the upper and lower respiratory tract epithelial cells [13]. Other animal studies have shown that both nasal and olfactory epithelial barriers were disrupted upon exposure to UPM [14,15].

The olfactory mucosa, which is located in the upper part of the nasal cavity closest to the cribriform plate, acts as the first line of defense against inhaled agents, including ambient particles [16]. Olfactory function is critical and important for both mice and humans. Mice can die from starvation without olfaction; in humans, olfaction can not only trigger an alarm due to dangers, such as fires, but also supports the sense of taste.

In our previous studies, the time to find food was significantly increased in the UPM group compared to the saline-treated group, revealing a difference between the UPM-treated and control groups (Supplementary data). Thus, we aimed to identify the factors affecting the destruction of the mouse olfactory system following UPM exposure.

We evaluated OMP, Olfr1507, ADCY3, and GNAL mRNA levels in the olfactory neuroepithelium, using RT-PCR [9,10]. We found the above-mentioned olfactory markers to be more decreased in the 200 UPM and 400 UPM groups than in the control group, at both 1 and 2 weeks. Furthermore, several olfactory markers were more decreased at 2 weeks than at 1 week.

We also evaluated various neurotrophic factors such as S100, CNPase, NGFRAP1, BDNF, and TACR3 mRNA levels [11], and found that these mRNA levels increased in the 200 UPM and 400 UPM groups compared to the control groups.

OECs have been used to improve axonal regeneration and functional outcomes in studies of spinal cord injury models [17]. Moreover, OECs have been studied in the context of peripheral nerves repair by direct transplantation in peripheral nerve lesion models [18]. Furthermore, olfactory neuroepithelial injury and regeneration could be intimately related to OECs owing to anatomical proximity [11]. In our previous studies, we reported that OEC was capable of regenerating the injured olfactory neuroepithelium [9,11]. However, if the OEC was injured, the activity of OEC could be decreased and the olfactory neuroepithelium could be damaged in conclusion. We hypothesized that the OECs could play a role in the destruction and recovery of the olfactory epithelium following olfactory neuroepithelial injuries. Therefore, we analysed the above mentioned OEC markers through gene and RNA expression analysis. The sensation of smell is also influenced by the somatosensory and chemesthetic sensations of the nose. Smells such as the cooling sensation of menthol or the prickle of carbon dioxide from carbonated drinks illustrate this point. These sensations are mediated in the nose by the trigeminal nerve, and there is increasing evidence that trigeminal and olfactory functions are closely linked and potentially interdependent [15]. Most odorants not only stimulate olfactory receptor neurons but also activate the intranasal trigeminal nerve. The simultaneous activation of the olfactory and the trigeminal system leads to an interaction in the brain [16]. TACR3 plays a protective role in treating trigeminal nerve injury-induced allodynia and anxiety-like behaviors [21]. Therefore, elevated TACR3 mRNA levels could indicate trigeminal nerve stimulation as additional support for olfactory neuroepithelium recovery following nasal tissue UPM exposure.

Finally, we dissected all the olfactory markers, OEC markers, and TACR3 using statistical analysis. The time (1 and 2 weeks) data showed that the correlation between the variables was positive, and that the correlation coefficient was highly significantly dif-

ferent from zero. We divided the 200 UPM and 400 UPM groups and analysed the relationship between the 2-week and 1-week groups. The correlation between the variables was highly positive in the 200 UPM groups, and the correlation coefficient was highly significantly different from zero. Thus, the duration of exposure might alter the change of the olfactory markers and the neurotrophic factors in the olfactory neuroepithelium.
The density could be another factor affecting the change in olfaction following UPM exposure. We demonstrated that the olfactory marker decreased significantly more severe at 200 UPM. The correlation score was not as high as that in the exposure time analysis. However, this could indicate that an appropriate density exists to change the olfaction. Further studies with various densities of UPM are required.
We analyzed the levels of the inflammatory markers: TNF-α, IFN-γ, IL-1, IL-5, IL-6, IL-10, IL-12, and IL-17 in the serum and nasal tissue, using PCR experiments, and found increased levels, usually at 1 week following UPM exposure. In gene studies, we found that the number of gene expressions related to the inflammatory and immune systems were elevated in the 400 UPM olfactory neuroepithelium. However, there were no statistically significant differences based on the analysis.
Although the changes in inflammatory mediators were significant in other studies, these studies usually use in vitro cell culture and treat the cells with UPM directly [22,23]. In this study, we used an animal model and administered the UPM to the nasal area, similar to that in the human environment. Some of the UPM could have been absorbed in the gastrointestinal and lower respiratory tracts, which could explain why the results differed between animal studies and cell culture studies owing to decreased bioavailability [24].

To summarise, we aimed to identify the influencing factors and the mechanism of changes following UPM exposure in the olfactory mucosa. First, the most important findings in this study are that the UPM dose and duration of exposure were moderately correlated with the mRNA expression and that the correlation was influenced by the olfactory system-related genes. Second, OEC activity might be crucial in stimulating the olfactory epithelium regeneration following UPM exposure. Moreover, the OEC markers could be affected by the exposure time and density of UPM. Third, the trigeminal nerve in the olfactory area may be related to olfactory destruction and regeneration; this is demonstrated by the significant increase in the substance P and TACR3 labeling densities in the UPM groups compared to the control group, in our immunolocalization and PCR study on the olfactory neuroepithelium. The simultaneous activation of the olfactory and the trigeminal system leads to an interaction in the brain [20]. So, if the trigeminal nerve in the olfactory area is injured, the olfactory function may be negatively impacted. On the other hand, activation of the trigeminal nerve might stimulate the olfactory function, potentially helping to regenerate the olfactory function.
Our study had several strengths. Our results reflect the actual bioavailability. We used an animal model in which the nose was exposed to UPM directly, similar to the actual human environment; in this context, it might provide realistic data. In contrast, some other studies [25,26], exposed cells to UPM which does not reflect the actual bioavailability. We therefore expect our study to provide a useful contribution to the field of olfactory science.
The limitation of our study was that this UPM model is not ideal for comparison with humans, because mice have a stronger regenerative ability than humans, and their olfactory epithelium recovers within 2 to 3 weeks of chemical injury [11-13]. However, due to ethical issues, it is logically more difficult to obtain clinical information from humans.

Conclusion

UPM could have cytotoxic effects on the olfactory neuroepithelium. The exposure time and density of the UPM could affect the degree of destruction of the olfactory neuroepithelium. The olfactory regeneration mechanism could be related to the olfactory receptor, neurotrophic factor, OEC stimulation, and trigeminal nerve sustenance. Therefore, it is likely that to improve the health of humans and prevent the destruction of olfactory function, environmental pollution and UPM must be reduced.

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Authorship contribution

Conceived and designed the manuscript: BYK, JHB. Analyzed the data: BYK, JYP. Contributed reagents/materials/analysis tools: BYK, KHC. Wrote the paper: BYK, JYP.

Conflict of interest

The authors claim that there are no conflicts of interest.

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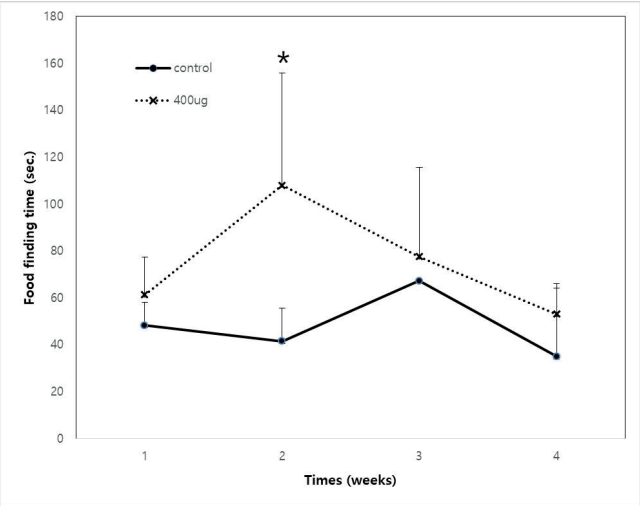
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. The food-finding tests were administered to each mouse before and after intranasal UPM administration. Mice in the control group found the food significantly faster than those in the 400UPM 2weeks group. *: p-value < 0.05, UPM: urban particulate matter.