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Melatonin inhibits the activation of microglia and cough sensitivity of guinea pigs exposed to PM2.5

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Summary. Objective. The study aimed to examine the impact of melatonin on mitigating brain inflammation and cough sensitivity resulting from exposure to particulate matter 2.5 (PM2.5).

Methods. Guinea pigs were randomly assigned to the blank control group, normal saline group, PM2.5 exposure group, and PM2.5 exposure + melatonin group. The PM2.5 exposure and PM2.5 exposure + melatonin groups were given intranasal instillations of PM2.5 suspension twice daily for 28 consecutive days. Starting on day 21, the PM2.5 exposure + melatonin group was treated with an intraperitoneal injection of melatonin at 10 pm. Cough sensitivity to citric acid, microglia activation, IL-1β and TNF-α levels in the airway and dorsal vagal complex (DVC), and ultrastructural changes in neurons within the DVC were assessed.

Results. The PM2.5 exposure group exhibited a significantly higher cough count to citric acid challenge (29.1±5.7 coughs) compared with the PM2.5 exposure + melatonin group (18.8±4.1 coughs), normal saline group (8.4±2.1 coughs), and blank control group (7.7±1.8 coughs). In addition, cough latency was shorter in the PM2.5 exposure group (26.9±6.5 seconds) than in the PM2.5 exposure + melatonin group (36.6±12.4 seconds), normal saline group (43.4±14.7 seconds), and blank control group (47.0±13.0 seconds). The PM2.5 exposure + melatonin group showed significantly reduced IL-1β (105.3±14.3 pg/ml) and TNF-α levels (113.0±23.5 pg/ml) in the DVC, as well as in the bronchoalveolar lavage fluid (IL-1β: 24.92±5.14 pg/ml, TNF-α: 12.72±3.99 pg/ml) compared with the PM2.5 exposure group (in the DVC: IL-1β: 132.7±17.6 pg/ml, TNF-α: 143.8±30.4 pg/ml; in the bronchoalveolar lavage fluid: IL-1β: 34.0±5.3 pg/ml; TNF-α: 15.8±0.8 pg/ml). Microglia in the DVC were less activated in the PM2.5 exposure + melatonin group (25.1±5.4) than in the PM2.5 exposure group (54.6±9.9). Furthermore, the PM2.5 exposure group exhibited an impaired blood-brain barrier in the DVC, which tended to alleviate the PM2.5 exposure + melatonin group.

Conclusions. Exposure to PM2.5 induces airway inflammation, central facilitation, and heightened cough sensitivity in guinea pigs. Melatonin significantly inhibits microglia activation and reduces airway and DVC inflammation, which might contribute to attenuated cough hypersensitivity.

Key words: PM2.5, Dorsal vagal complex, Inflammation, Central facilitation, Cough sensitivity

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Introduction

Cough is a crucial protective reflex in the human body and represents a prevalent symptom of respiratory illnesses. Persistent, recurring coughing significantly diminishes individuals' overall quality of life (Martin Nguyen et al., 2021). Cough typically occurs reflexively by activation of the airway sensory fibers of the vagus nerve that terminate peripherally near the epithelium in the larynx, trachea, as well as large intrapulmonary bronchi, and centrally in the brain stem, which encompasses both the nucleus of the solitary tract and paratrigeminal nucleus (Canning et al., 2004; Mazzone and Undem, 2016). Chronic cough of any etiology is

widely believed to reflect a hypersensitivity condition characterized by coughing often triggered by low levels of thermal, mechanical, or chemical exposure (Chung et al., 2022). Multiple interrelated mechanisms have been hypothesized to contribute to cough hypersensitivity. These mechanisms include increased activation or excitability of sensory neurons to stimulation in the periphery, or the heightened responsivity of central cough networks to peripheral inputs, respectively termed peripheral and central sensitization (Mazzone and McGarvey, 2021). Inflammatory mediators released from an array of resident or infiltrating cells within the inflamed or injured airway and cough center were thought to take part in the sensitization of the cough reflex. In patients with problematic cough, airway epithelium, resident fibroblasts, vascular endothelium, airway wall smooth muscle cells, platelets, lymphocytes, macrophages, mast cells, and even the neurons themselves and their associated glial cells were found to be involved (Chung et al., 2013; McGovern and Mazzone, 2014; Undem and Taylor-Clark, 2014).

Particulate matter (PM) is a complex mixture of solids and liquids suspended in the air. In recent years, more and more attention has been paid to the impact of PM pollution on public health. Among these, particles less than 2.5 micrometers in diameter, also known as fine particles or PM2.5, pose the greatest health risk (Lin et al., 2022; Cheng et al., 2024). A direct association has been found between PM2.5 and chronic cough (Wang et al., 2018). PM2.5 has a large surface area which helps it absorb various harmful substances and, because of its small particle size, it can penetrate deep into the lungs and deposit in the terminal bronchioles and alveoli with the breath, subsequently inducing airway inflammation (Yang et al., 2020b). There is a growing focus on the influence of PM2.5 on the central nervous system. Animal studies have demonstrated that PM2.5 can invade the central nervous system via the olfactory pathway to induce neurotoxicity (Kim et al., 2021; Wei et al., 2024). Microglia activation and the release of inflammatory mediators might contribute to the neuroinflammation caused by PM2.5 (Kim et al., 2020; Chen et al., 2021; Jin et al., 2021).

Melatonin has been shown to protect against PM2.5-induced lung injury (Guohua et al., 2021) and suppress inflammation in the central nervous system and blood-brain barrier disruption (Ji et al., 2018; Thangwong et al., 2023). Nevertheless, whether it can alleviate PM2.5-induced cough remains unknown. We speculated that melatonin has the potential to attenuate cough hypersensitivity caused by PM2.5 exposure by diminishing the release of inflammatory mediators within the airway and dorsal vagal complex (DVC).

Materials and methods

PM2.5 collection and suspension preparation

As described in our previous study (Lv et al., 2016), to acquire representative samples, PM2.5 was collected at five sites in Nanjing city by the urban function: Hunan Road, Maigaoqiao, Shanxi Road, industrial area along the Yangtze River and Xianlin University which represented business areas, commercial areas, heavy traffic areas, industrial areas, and the suburbs, respectively. Sampling was conducted 8h per day for 90 days, using a TH-150C atmospheric intelligent sampler equipped with a PM2.5 cutting head, set at a height of 1.5 meters and a flow rate of 3.5 L/min. Following initial sampling, the filter membrane was immersed in deionized water and subjected to 30 minutes of ultrasonic agitation to extract particles. This was followed by filtration, drying, and assessment of particle dispersion. The components of PM2.5, which were identified using the receptor model chemical mass balance method, were as follows (presented as mean±SEM, %): organic carbon (35.3±3.4), elemental carbon (31.7±4.7), Cu (4.3±1.6), Fe (2.4±1.5), Al (5.8±3.2), Ca (3.2±2.0), inorganic acid ion (6.7±1.4), biological substance (9.4±5.3), and others (2.2±2.1). The sample was then resuspended in sterile saline to obtain a working solution of 0.1 g/ml and stored in a refrigerator at 4°C.

Animal model

Male albino guinea pigs (age: 10-12 weeks, body weight: 350-400 g) were allocated randomly into four groups (n=24 for each group): blank control, normal saline, PM2.5 exposure, and PM2.5 exposure + melatonin (melatonin intervention group). According to a previous study, guinea pigs in the PM2.5 exposure group were given 200 μl intranasal instillations of PM2.5 suspension (containing 20 mg PM2.5) at 12 am and 8 pm daily for 28 consecutive days. The normal saline group was given intranasal instillations of sterile normal saline, following the same dosage and timing as the PM2.5 exposure group. In the melatonin intervention group, animals underwent the identical instillation protocol as the PM2.5 exposure group. Starting on the 21st day, melatonin (10 mg/kg) was given via intraperitoneal injection at 10 pm for 7 days. The blank group was housed in the same conditions without any intervention, functioning as a control to assess the impact of the housing environment and intranasal procedures. All guinea pigs were housed in a standard environment with a temperature of 20 to 24°C, a humidity of 50 to 60% under a 12-hour light-dark cycle, and provided with food and water without limitation. All procedures for animal care and use followed the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the and Use of Laboratory, 2011) and approved by the Institutional Animal Care and Use Committee of Jiangsu University.

Cough sensitivity test

Cough sensitivity tests were performed a day before modeling (day 0), and a day after modeling (day 29). A

402AI ultrasonic nebulizer with an output flow rate of 37 ml/min was utilized. Guinea pigs were placed in individual transparent Perspex chambers and exposed to a nebulized aqueous solution of 0.45 M citric acid for 15 seconds, during which cough responses were monitored by the PowerLab biological signal acquisition and processing system. The time elapsed from the initiation of stimulation to the onset of the first cough (cough latency), and the total number of coughs (cough counts) within a three-minute timeframe, were recorded as parameters to assess cough sensitivity.

Detection of cytokines in bronchoalveolar lavage fluid

Guinea pigs were anesthetized via intraperitoneal injection of 10% chloral hydrate (50 mg/kg). Subsequently, the trachea was exposed and the endotracheal tube was inserted. The thoracic cavity was then opened, and the right hilum of the lung was ligated with thread, a total of 3 ml of pre-cooled normal saline was gradually administered into the airway and lungs through the endotracheal tube, and withdrawn after 30 seconds and then injected again, repeated three times. The withdrawal rate was around 70%, and the liquid obtained was injected into a 10 ml plastic centrifuge tube placed in an ice bath, which was subsequently centrifuged at 3000 rpm/min for 10 minutes at 4°C. The supernatant was used for TNF-α and IL-1β detection by ELISA kits (n=8 for each group).

Detection of cytokine levels in the brain

Following bronchoalveolar lavage, the guinea pig's brain was removed. The brain regions were taken and rinsed in ice-cold saline to remove blood, then dried with filter paper. Pre-cooled normal saline equivalent to nine times the weight of the tissue was added and mixed with the brain tissue in a homogenization tube. All procedures were performed on ice. The homogenate was subsequently centrifuged at 3000 rpm/min for 15 minutes at 4°C. The supernatant was used for TNF-α and IL-1β detection by ELISA kits (n=8 for each group).

HE staining and immunofluorescence

The brain, trachea, and lungs for hematoxylin and eosin (HE) staining and immunofluorescence were collected from guinea pigs who had not undergone bronchoalveolar lavage (n=8 for each group). After normal saline perfusion and 4% paraformaldehyde fixation, the brain, trachea, and lungs were removed and stored in 4% paraformaldehyde for 12h. The brain tissue was then transferred to 30% sucrose for dehydration. The morphological changes in lung tissue were assessed using HE staining. Dehydrated brain tissue was sliced into 30-μm-thick frozen sections, treated with 3% hydrogen peroxide for 15 minutes, and rinsed with phosphate-buffered saline (PBS) three times for five minutes each time. It was then blocked with goat serum for 30 minutes before incubation with anti-OX-42 primary antibody (1:400, mouse antibody, Abcam) overnight, then washed with PBS containing 0.3% Triton X-100 three times for five minutes each time. Subsequently, it was incubated with fluorescein-labeled secondary antibody (1:200, goat anti-mouse, Abcam) for 1h, and washed with PBS three times. Examinations were performed by a fluorescence microscope, and the immunopositive cells were quantified by Image-ProPlus 6.0.

Transmission electron microscope

After normal saline perfusion, the guinea pigs were fixed by perfusion with pre-cooled 4% paraformaldehyde + 0.5% glutaraldehyde phosphate buffer for 15 minutes, and the brain was quickly removed on ice. A 1-mm-thick slice was scraped from the selected nucleus with a sharp blade in 4% paraformaldehyde + 0.5% glutaraldehyde phosphate buffer. Then three pieces with a volume of 1 mm3 were cut from the slice, and placed in a 1.5 ml EP tube containing 2.5% glutaraldehyde for 4 hours. Following PBS washing, the specimens underwent fixation with 1% osmium tetroxide for 1.5 hours, dehydration in a series of acetone solutions, and then resin infiltration and embedding before being sectioned into 80-nm slices using an ultramicrotome. The sections were then subjected to double staining with uranium and lead, and examined under a transmission electron microscope (HITACHI, H-7650). Five random regions were chosen on each section at an equivalent magnification. The ultrastructural alterations of the blood-brain barrier, nerve cells, and

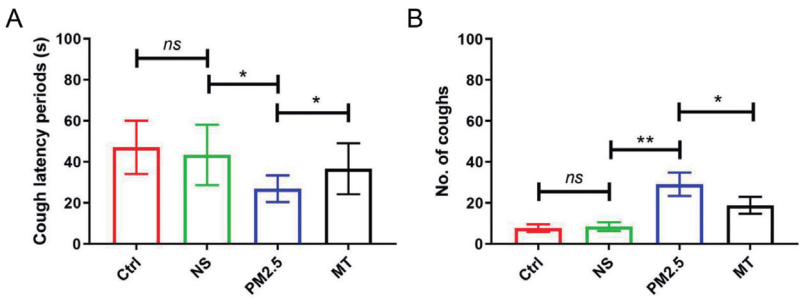

Fig. 1. Changes in cough sensitivity. **A.** Cough latency of guinea pigs. **B.** Cough counts of guinea pigs. Ctrl: blank control group, NS: normal saline group, PM2.5: PM2.5 exposure group, MT: PM2.5 exposure + melatonin group. *p<0.05, **p<0.01. N=8 for each group.

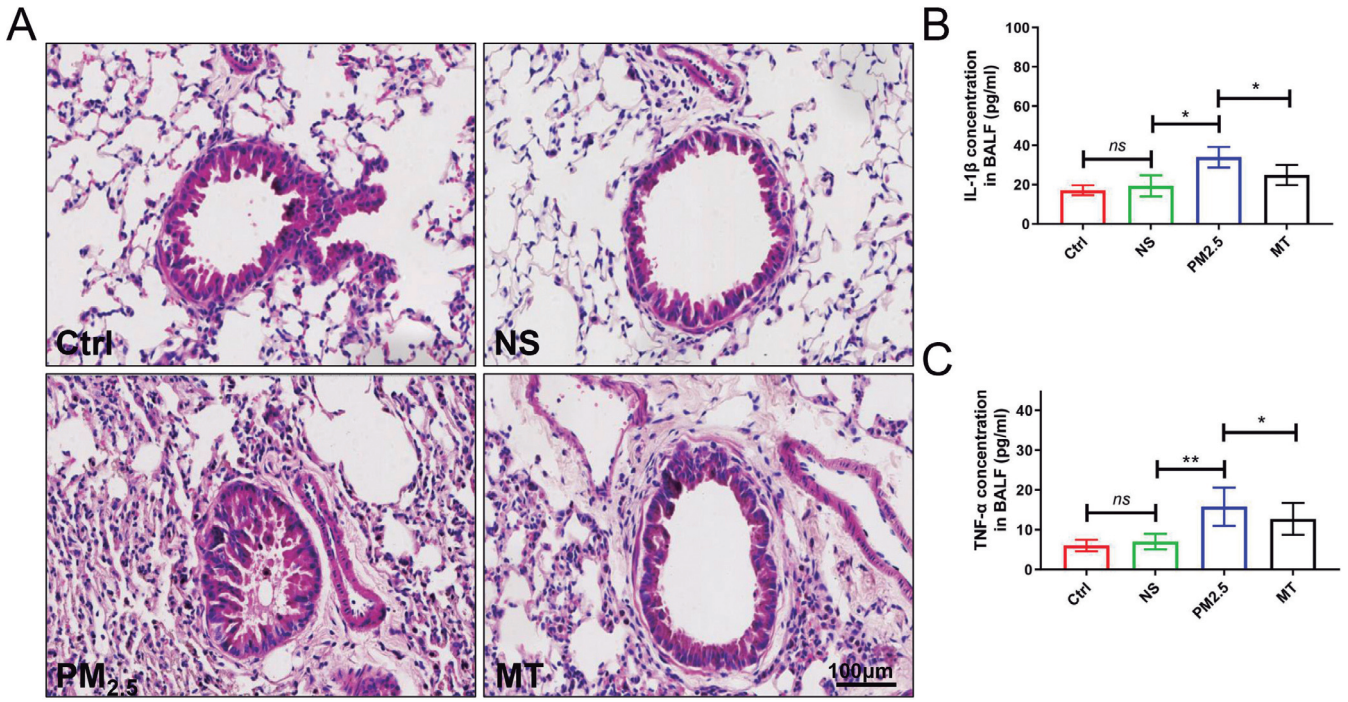

Fig. 2. Melatonin alleviates airway inflammation induced by PM2.5 exposure in guinea pigs. **A.** HE staining of lung tissue. **B.** IL-1 β in bronchoalveolar lavage fluid of guinea pigs. **C.** TNF- α in bronchoalveolar lavage fluid of guinea pigs. Ctrl: blank control group, NS: normal saline group, PM2.5: PM2.5 exposure group, MT: PM2.5 exposure + melatonin group. BALF: bronchoalveolar lavage fluid. *p<0.05, **p<0.01. N=8 for each group.

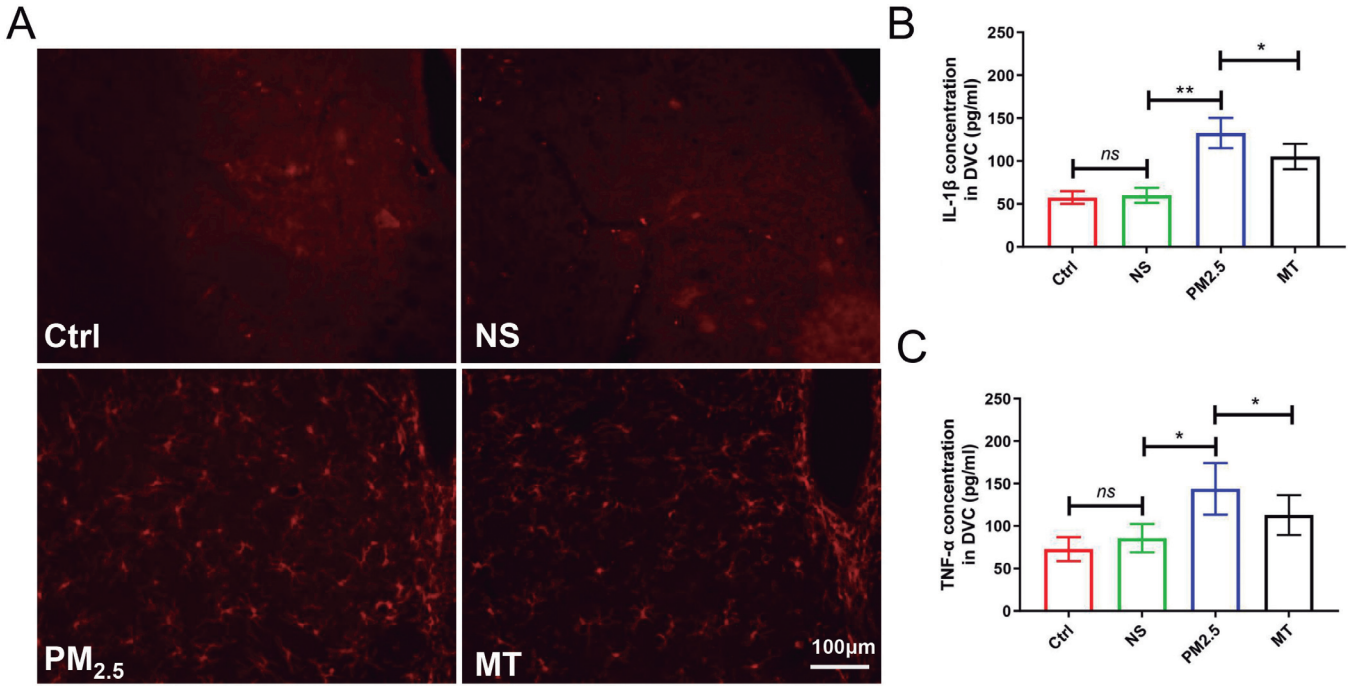

Fig. 3. Melatonin reduces microglial activation and inflammation in the dorsal vagal complex caused by PM2.5 exposure. **A.** Immunofluorescence of OX-42 in the DVC. **B.** IL-1β level in the DVC. **C.** TNF-α level in the DVC. Ctrl: blank control group, NS: normal saline group, PM2.5: PM2.5 exposure group, MT: PM2.5 exposure + melatonin group. DVC: dorsal vagal complex. *p<0.05, **p<0.01. N=8 for each group.

nerve fibers were assessed, and the fine particles in each region were quantified.

Statistical analysis

Statistical analysis was performed by SPSS 18.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean±SD. ANOVA was used for comparisons among groups. For pairwise comparisons, the LSD test was used if the data conformed to the assumption of homogeneity of variance, otherwise, the Games-Howell test was applied. A $p<0.05$ was considered statistically significant.

Results

Changes in cough sensitivity

The PM2.5 exposure group exhibited a significantly higher cough count to citric acid challenge (29.1±5.7 coughs) compared with the PM2.5 exposure + melatonin (18.8±4.1 coughs), normal saline (8.4±2.1 coughs), and blank control groups (7.7±1.8 coughs). In addition, the cough latency was significantly shorter in the PM2.5 exposure group (26.9±6.5 seconds) than in the PM2.5 exposure + melatonin (36.6±12.4 seconds), normal saline (43.4±14.7 seconds), and blank control groups

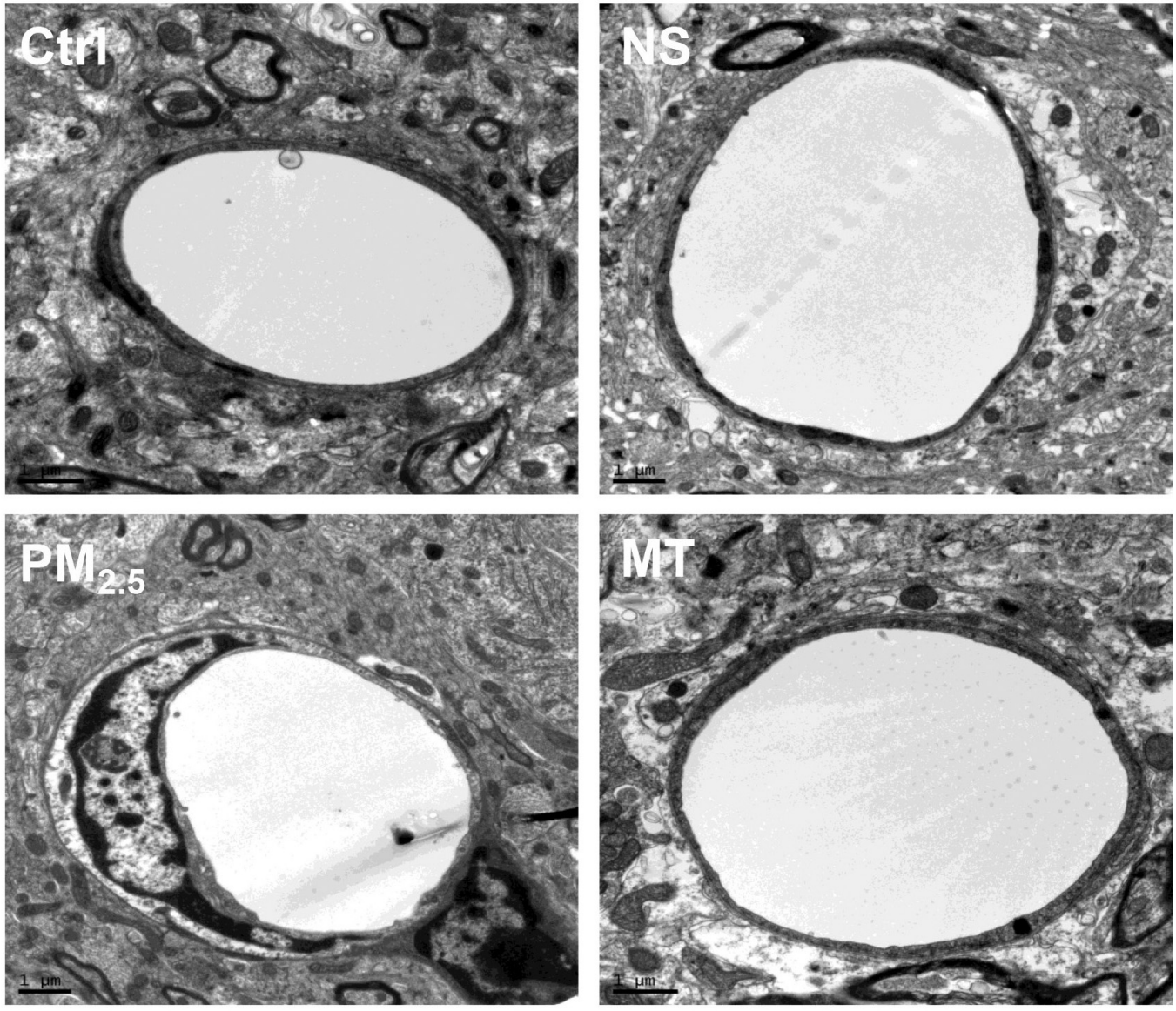

Fig. 4. Transmission electron microscopy results of the blood-brain barrier in the medulla-vagal complex. Ctrl: blank control group, NS: normal saline group, PM2.5: PM2.5 exposure group, MT: PM2.5 exposure + melatonin group. N=8 for each group.

(47.0±13.0 seconds). No significant difference in cough frequency or latency was observed between the normal saline and the blank control groups (Fig. 1).

Airway inflammation

Neither the blank control group nor the normal saline group exhibited any abnormalities in their airway and lung tissues. The PM2.5 exposure group displayed neutrophil and eosinophil infiltration within the airway and lung tissues, while decreased neutrophils and eosinophils were observed in the PM2.5 exposure + melatonin group (Fig. 2A). The PM2.5 exposure group (34.0±5.3 pg/ml) showed the highest IL-1β level in the bronchoalveolar lavage compared with the PM2.5 exposure + melatonin (24.9±5.1 pg/ml), normal saline (19.4±0.4 pg/ml), and blank control groups (17.1±0.5 pg/ml) (Fig. 2B). The TNF-α level in the bronchoalveolar lavage was also elevated in the PM2.5 exposure group (15.8±0.8 pg/ml) compared with the PM2.5 exposure + melatonin (12.7±2.0 pg/ml), normal saline (7.0±0.5 pg/ml), and blank control groups (6.0±0.4 pg/ml) (Fig. 2C). No significant differences in IL-1β or TNF-α levels were observed between the normal saline and blank control groups.

Activation of microglia cells and inflammation in the dorsal vagal complex

More microglia cells were activated in the PM2.5 exposure group (54.6±9.9) than in the PM2.5 exposure + melatonin (25.1±5.4), normal saline (3.5±1.4), and blank control groups (5.1±1.2) (Fig. 3A). The PM2.5 exposure group (132.7±17.6 pg/ml) showed the highest IL-1β level in the DVC compared with the PM2.5 exposure + melatonin (105.3±14.8 pg/ml), normal saline (60.1±8.7 pg/ml), and blank control groups (57.4±7.4 pg/ml) (Fig. 3B). The TNF-α level in the DVC was also elevated in the PM2.5 exposure group (143.8±30.4 pg/ml) compared with the PM2.5 exposure + melatonin (113.0±23.5 pg/ml), normal saline (75.7±16.7 pg/ml), and blank control groups (72.8±14.2 pg/ml) (Fig. 3C). No significant differences in IL-1β or TNF-α levels were observed between the normal saline and the blank control groups.

Damage to the blood-brain barrier

PM2.5 exposure guinea pigs exhibited vascular endothelial swelling, basement membrane loosening, astrocyte vascular endfeet activation, and endothelial cell gap expansion, suggesting compromised blood-brain barrier integrity and heightened permeability, which were much less in guinea pigs treated with melatonin. Neither the blank nor the normal saline groups showed any observable damage to the blood-brain barrier (Fig. 4).

Discussion

Exposure to PM2.5 is known to be associated with the onset of chronic cough (Doiron et al., 2021), however, the underlying mechanisms remain to be elucidated. This study successfully established a PM2.5-induced chronic cough model in guinea pigs and found that intranasal instillation of a PM2.5 suspension not only results in airway inflammation but also microglial cell activation in the brain, initiation of brain inflammation, and disruption of the blood-brain barrier. Melatonin, an antioxidant, can alleviate the

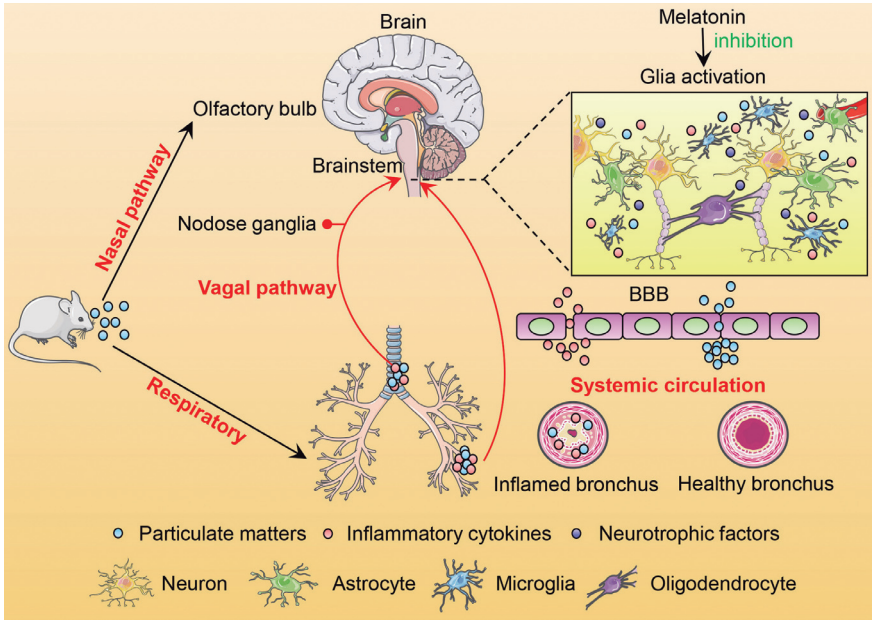

Fig. 5. Mechanisms of PM2.5-induced cough hypersensitivity and antitussive potential of melatonin

inflammatory response in both the airway and the central nervous system, thereby, reducing cough hypersensitivity.

PM2.5 can destroy the integrity of the blood-brain barrier, so peripheral systemic inflammation easily crosses the blood-brain barrier and reaches the central nervous system, and the olfactory nerve is another way for PM2.5 particles to enter the brain (Shou et al., 2019). It used to be believed that the adult brain is protected from air pollutants by the blood-brain barrier. As research on air pollutants advances, it has been discovered that PM2.5 can permeate the blood-brain barrier and accumulate in the brain tissue; astrogliosis is then initiated and results in microglial infiltration (Kang et al., 2021; Qin et al., 2024). Microglia are the major innate immune cells of the brain, surveilling and maintaining the homeostasis of the central nervous system. Upon activation by environmental and endogenous insults, such as PM exposure, microglia can enter an overactivated state that is characterized by amoeboid morphology, the over-production of reactive oxygen species, and pro-inflammatory mediators (Song et al., 2022). The strong link between PM2.5 and microglia-derived neuro-inflammation has been established in neurodegenerative diseases, such as Alzheimer's disease, however, its role in cough hypersensitivity remains unclear.

We speculate that there are two ways by which PM2.5 can induce cough hypersensitivity. On the one hand, inhaled PM2.5 deposits in the airway, damages the airway epithelium and vagal fibers, causing peripheral neurogenic inflammation. In this condition, cough receptors are more susceptible to mechanical and chemical stimuli, hence, signals are frequently transmitted from vagal nerves to the cough center, resulting in central sensitization and facilitation. On the other hand, PM2.5 can reach the central nervous system through the blood-brain barrier and olfactory nerve, activate microglia that over-produce reactive oxygen species and pro-inflammatory mediators in the DVC, contributing to central sensitization and facilitation, thereby inducing cough hypersensitivity. Melatonin, a neuroendocrine hormone produced by the pineal gland, can attenuate blood-brain barrier disruption by increasing tight junction proteins, inhibiting microglia activation, and alleviating the inflammatory response and oxidative stress in the central nervous system (Yang et al., 2020a; Chen et al., 2022; Thangwong et al., 2023). In addition, animal studies have confirmed that melatonin can reduce airway inflammation and hyperreactivity (Chen et al., 2011; Peng et al., 2018). These findings make melatonin a potential candidate for treating cough hypersensitivity (Fig. 5).

In conclusion, exposure to PM2.5 induces airway inflammation, central facilitation, and heightened cough sensitivity in guinea pigs. Melatonin significantly inhibits microglia activation and reduces airway and DVC inflammation, which might contribute to attenuated cough hypersensitivity.

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Competing interests. There is no conflict of interest in this manuscript.
Consent for publication. The manuscript is approved by all authors for publication.
Availability of data and materials. The data and materials of this experiment are available.
Author contributions. Shu Zhang and Li Long were responsible for the conception and design. Senlin Chai, Mingtong Lin, Yaowei He, Hankun Lu, and Xuemei Liu were responsible for the collection and assembly of data. Rong Dong and Zhe Chen were responsible for data analysis and interpretation. All authors were responsible for manuscript writing and the final approval of the manuscript.
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