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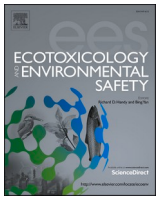

A molecular toxicological study to explore potential health risks associated with ultrafine particle exposure in cold and humid indoor environments

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ABSTRACT

Environmental pollutants including ultrafine particulate matter (UFPs) and adverse meteorological conditions pose significant public health impacts, particularly affecting respiratory health. This study aims to elucidate the synergistic effects of cold-humid conditions and UFPs exposure on respiratory health, utilizing Carbon Black Nanoparticles (CB-NPs) as surrogates for UFPs. Through comprehensive lung function tests, histopathological examinations, and biomarker analyses, this research focuses on the modulation of oxidative stress signaling pathways and NF-κB activation. Male Balb/c mice were exposed to specific concentrations of CB-NPs (30–50 nm in diameter, 0.184 mg/(kg·day)) in a controlled environmental chamber mimicking cold (10°C/14°C) and humid (90 % RH) conditions over three weeks. The results indicate that exposure to CB-NPs alone increased lung function, oxidative stress (ROS, GSH, MDA), inflammation (IL-6, TNF-α, IL-1β), apoptosis (Caspase 3, Caspase 8, Caspase 9), and histopathological alterations in lung tissue. Furthermore, these effects were notably more severe under combined exposure with cold-humid conditions. These results suggest that the adverse effects of pollutants are not solely concentration-dependent but are exacerbated by specific environmental contexts. It is evident that Vitamin E (100 mg/kg/day) can attenuate these adverse effects, underscoring its potential as a protective agent against environmental stressor-induced air pollutants and cold humid conditions. Our findings suggest that the synergistic effects of environmental factors and pollutant exposure significantly impact respiratory health, providing valuable insights for the design of healthier indoor environments and the development of strategies to mitigate these risks.

1. Introduction

Air pollution poses a significant environmental risk to public health. This risk is compounded considering that individuals typically spend about 80 %-90 % of their time indoors (ASHRAE, 2010). With the rapid development of society and improvements in technology, the research on indoor air pollution has further deepened our understanding (Manisalidis et al., 2020; Noël et al., 2021; Ukaogo et al., 2020). Indoor air pollutants include physical pollution (e.g. particulate matter, dust), chemical pollution (e.g. organic volatiles such as formaldehyde), and biological pollution (e.g. molds, fungi) which impact ambient air quality as well as thermal and humid environments, acoustic environments, and light environments (Tran et al., 2020).

In 2019, air pollution caused 4.9 million deaths worldwide based on the global burden of death (GBD). A key component of this pollution is particulate matter, which the World Health Organization's Agency for Research on Cancer (IARC) has identified as a carcinogen (Loomis et al., 2013). Due to diversities in its diameter and composition type, particulate matter (PM) may impose multiple effects on the whole body especially in the cardiovascular and respiratory system, such as causing systemic inflammation and oxidative stress, blood pressure increases, or changes in serum metabolites (Manisalidis et al., 2020; Ali et al., 2022;

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Basith et al., 2022; Lakhdar et al., 2022; Shahrbaf et al., 2021; Shahriyari et al., 2022). The biological effects of ultrafine particulate matter (UFPs), including carbon-based nanoparticles (CB-NPs) are made more important by its inherent small particle size, large specific surface area, and high quantitative concentrations (Chen et al., 2016; Kumar et al., 2014). CB-NPs are a significant subset of UFPs and are widely emitted from both indoor sources, such as heating, cooking, and printing, and outdoor sources, such as vehicle exhaust and industrial emissions (Kuye and Kumar, 2023). CB-NPs not only act as a carrier for toxic substances but also serve as environmental adjuvants that exacerbate allergic sensitization and airway inflammation (Kroker et al., 2015). The inhalation pathway is the main route to UFPs exposure, and hence causes the most significant effects on human health; whereas, dermal, ocular, and nasal mucosal contacts account for lower exposure (Chen et al., 2016). During respiration, coarse particulate matter is deposited mainly in the nasal cavity as well as in the upper respiratory tract, while ultrafine particulate matter is deposited mainly in the trachea, bronchi and alveoli of the lungs (Mack et al., 2019). The lungs, as the main deposition site for ultrafine particulate matter, are an important target organ for toxic effects.

Cold and humid physical environments are equally capable of affecting human lung function health. Numerous studies have shown that temperature and health are interconnected in a V- or U-shaped pattern, with a decline in health risks until a certain inflection point is reached (Liu et al., 2022; Rizmie et al., 2022). Cold is known to exacerbate airway inflammation and potentially impair lung function (Cockcroft and Davis, 2006), inducing a potential risk of respiratory (Wolkoff et al., 2021; Han et al., 2023), cardiopulmonary (Zafeiratou et al., 2021), and neurological disorders, as well as increased mortality (Ma et al., 2020; Xu et al., 2022; Zhang et al., 2016). Decreased temperatures have been linked to a higher incidence of chronic obstructive pulmonary disease (COPD) (McCormack et al., 2017; Qiu et al., 2018), and variations in indoor temperature are associated with short-term changes in lung function among children with asthma (Pierse et al., 2013). Lower temperature is not only detrimental to the lung health of people with underlying conditions, but also has negative health effects on the lungs of healthy people (Qiu et al., 2023). A healthy volunteer natural migration (HVNR) study suggests that exposure to low temperatures could lead to a reduction in lung function (Wu et al., 2014). In addition, high indoor humidity levels are also identified as a risk factor for adverse lung health (Jaakkola et al., 2010; Norbäck, 2020). A meta-analysis has demonstrated an increase in respiratory and asthma-related health risks by 30–50 % due to building dampness and mold (Fisk et al., 2007). Additionally, residential dampness and mold were significantly associated with an increase in respiratory infections and bronchitis (Fisk et al., 2010). A study focusing on lung function and living environments of European adults aged 20–45 years revealed that high humidity is a contributing factor to reduced lung function, thereby posing a health risk (Norback et al., 2011).

Recent evidence suggests that co-exposure to various air pollutants can synergistically amplify their individual effects (Sava and Carlsten, 2012; Wang et al., 2023). Moreover, the behavior of UFPs in the environment, such as their residence time and diffusion, is significantly influenced by changes in temperature and relative humidity (Xiao et al., 2020; Yuan et al., 2023). Studies conducted in regions of China with extreme seasonal temperatures have revealed that indoor environments during winter are particularly harsh, often characterized by low temperatures and high relative humidity (Zhang and Yoshino, 2010; Li et al., 2023; Liu et al., 2017). Exposure to both cold, damp conditions and UFPs is common, especially in winter due to factors like smoking and cooking activities (Abdillah and Wang, 2023). Considering the possible effects of colder-damp environments and UFPs on lung injury, it is necessary to explore their combined effects on lung function, which is particularly important for assessing potential risks to lung function in healthy individuals. Epidemiological time series studies have shown that environmental factors including changes in temperature and humidity that influence particulate matter concentrations are associated with increased hospitalizations for respiratory and cardiovascular diseases (Klompmaker et al., 2021; Tsao et al., 2023; Wu et al., 2022), and this effect could be exacerbated by elevated levels of black carbon (Lepeule et al., 2018a). A small number of toxicological studies investigating the effects of exposure to larger particulate matter ($PM_{2.5}$) under cold stress conditions have suggested enhanced toxic effects in the lungs of rats (Luo et al., 2014). However, given that UFPs may have greater potential for adverse health effects, there is still a lack of co-exposure studies to confirm hypotheses and explain the biological mechanisms by which these affect human health (Kumar et al., 2016; Moreno-Ríos et al., 2022). Since cold temperature and high humidity environments are widely distributed and there are many sources of CB-NPs, scenarios involving co-exposure to these elements are likely to be common. Existing research provides some insights, but gaps remain in understanding the combined impact of cold temperature/high humidity/CB-NPs on pulmonary function and pathology.

Oxidative stress damage in lungs is a well-documented mechanism in ultrafine particle studies, particularly involving the mediation of reactive oxygen species (ROS). The activation of NF-κB by ROS is hypothesized to amplify airway inflammation (Leikauf et al., 2020). Based on preliminary research and literature, we propose that if a synergistic effect exists in combined exposure scenarios, it may operate through a cascade of oxidative stress leading to inflammation-mediated cellular apoptosis.

This study aims to explore the potential health risks associated with ultrafine particles exposure in cold and humid indoor environments during winter. This is the first to provide pioneering insights into the synergistic effects of ultrafine particles and two climatic stressors on respiratory health. Based on environment-related health-oriented molecular toxicology, the research strategy of this work examines 'environment stress, key molecular events, impact on lung tissue, and end effects of respiratory conditions'. To achieve the aim, the following objectives were established: 1) to conduct animal exposure experiments in different indoor climatic conditions alongside the particulate matter exposure limits set up by the current indoor environment design standard; 2) to investigate the impact of individual and combined indoor air pollutants on lung health; 3) to evaluate pulmonary function and analyze histopathological lung sections; and 4) to investigate the potential protective and therapeutic role of antioxidants in mitigating lung injury. This study will provide new insight into the joint effects of chemical and physical risks on respiratory health. The results will serve as a reference for assessing the health risks of exposure to climatic stress and ultrafine particles thereby informing guidelines for indoor air quality management and public health policy.

2. Materials and methods

2.1. Experimental animals and ethical review

Specific pathogen-free (SPF) 6–8 weeks old male Balb/c mice were obtained from the Hubei Provincial Center for Disease Control and Prevention Experimental Animal Center, with animal certificate No. 42000600043994. The mice were acclimated to standard environmental conditions, including a 12-hour light-dark cycle, 50–60 % humidity, and a temperature of 20–25°C. They were provided with sufficient food and water in the animal care facility prior to environmental exposure. All experimental procedures followed were approved by The Academic Ethics Supervision Committee of Hubei University of Science and Technology, with the approval ID HBUST-IACUC-2021–002.

2.2. The risk factor exposures and animal grouping

Hygrothermal conditions were established utilizing animal incubators (RDW-600B-3) from Ningbo Southeast Instrument Co., Ltd., China. These conditions encompassed a standard relative humidity level

of 50–60 % and an elevated level of 90 %. In the latter environment, mice were continuously subjected to 90 % relative humidity over 12-hour periods at a consistent temperature of 25℃ (Duan et al., 2020). According to the size distribution characteristics of environmental ultrafine particles (Ihrie and Bonner, 2018; Li et al., 2016), Carbon Black Nanoparticles (CB-NPs) with a representative size of 30–50 nm were purchased from XFNANO Materials Tech Co., Ltd (Jiangsu, China), see the characterization diagram in Fig. 1. These CB-NPs derived from coal tar were sterilized for standby after purchase. Carbon Black Nanoparticles (CB-NPs) were prepared using sterile physiological saline enriched with 0.05 % Tween-80. After bath sonication of this solution, the mice were intranasally instilled with the nanoparticles (Park et al., 2015; Ronzani et al., 2014; Deng et al., 2022a). The dosage of CB-NPs administered daily was derived using the equation Y = (12•X)/60. Here, 12 m^3/day represents the average daily air intake and 60 kg signifies the typical body weight of an adult. The term X is defined as the air particle concentration (μg/m^3). According to China's Ambient Air Quality Standards (GB 3095–2012), the 24-h average permissible concentration for $PM_{2.5}$ (with an aerodynamic diameter < 2.5 μm) stands at 75 μg/m^3. Utilizing this standard, with X being 75 μg/m^3, Y was determined as 15 μg/(kg•day).

The exposure matrix of hygrothermal environment (cold-humid) and/or CB-NPs to mice is presented in Table 1. A total of 156 mice weighing between 180 and 220 g each were randomly divided into twelve groups, with 13 mice in each group. The groups were as follows: (1) control group treated with 0.9 % saline (Control), (2) VE control group treated with VE 100 mg/kg bw (VE), (3) the group exposed to CB-NPs at a dose of 15 μg/(kg•day) bw (CB), (4) the group exposed to 10℃ temperature with normal humidity (LT), (5) the group exposed to 14℃ temperature with normal humidity (HT), (6) the group exposed to 10℃ temperature with 90 % high humidity (LTH), (7) the group exposed to 14℃ temperature with 90 % high humidity (HTH), (8) the group exposed to CB-NPs at a dose of 15 μg/(kg•day) bw with 10℃ temperature and normal humidity (CB-LT), (9) the group exposed to CB-NPs at a dose of 15 μg/(kg•day) bw with 14℃ temperature and normal humidity (CB-HT), (10) the group exposed to CB-NPs at a dose of 15 μg/(kg•day) bw with 10℃ temperature and high humidity (CB-LTH), and (11) the group exposed to CB-NPs at a dose of 15 μg/(kg•day) bw with 14℃ temperature and high humidity (CB-HTH). After evaluating the level of lung inflammation, the group with the most severe combined exposure, CB-LTH, was further treated with 100 mg/kg bw VE and denoted as CB-LTH+VE (12). The doses of CB-NPs and VE were determined based on the daily exposure and ingestion of the general population.

The mice were administered CB-NPs suspensions or 0.9 % saline daily from 08:00–09:00 via intratracheal instillation for a total of 21 days. The volume of instillation was 0.1 ml/100 g bw. Intragastric administration of Vitamin E (VE) took place 4 hours after the intratracheal instillation. Five mice from each group were used to measure airway hyperresponsiveness (AHR). The remaining 8 mice in each group were used for lung histological assay and biomarker analysis. The detailed experimental protocol is shown in Fig. 2.

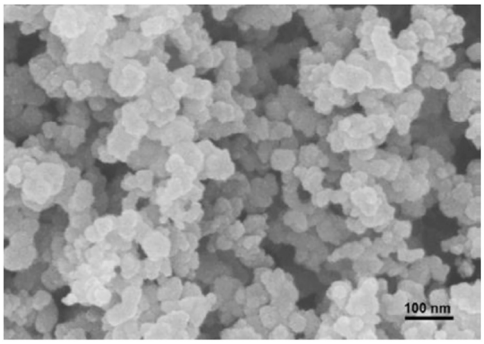

Fig. 1. SEM graph of Carbon Black Nanoparticles (CB-NPs) sample.

Table 1
Exposure matrix of hygrothermal environment (cold-humid) and/or CB-NPs 15 μg/kg•day).

Group	Treatment					
	Temperature			Relative humidity		CB-NPs*
	10°C	14°C	20–25°C	50 %	90 %	+ or /
Control	/	/	+	+	/	/
CB	/	/	+	+	/	+
LT	+	/	/	+	/	/
HT	/	+	/	/	/	/
LTH	+	/	/	+	/	/
HTH	/	+	/	/	+	/
CB-LT	+	/	/	+	/	+
CB-HT	/	+	/	+	/	+
CB-LTH	+	/	/	/	+	+
CB-HTH	/	+	/	/	+	+

2.3. Measurement of airway hyperresponsiveness

In this study, we assessed airway hyperresponsiveness (AHR), which is known to be associated with an increased risk of respiratory symptoms and diseases, including asthma. To measure AHR, we recorded airway resistance using Methacholine (MCH) with the AniRes2005 Lung Function System (Bestlab ver2.0; Beijing, China). Prior to the experiment, the mice were anesthetized with 10 % pentobarbital sodium and respiration was maintained by tracheal intubation with a small animal ventilator (exhalation rate and the expiration/inhalation time ratio were preset to 90/min and 1.5:1.0, respectively). MCH was injected via a catheter at 5-minute intervals, with doses of 0.025, 0.05, 0.1, and 0.2 mg/kg bw. The AniRes2005 Lung Function System automatically recorded the expiratory resistance (Re), inspiratory resistance (Ri), and dynamic lung compliance (Cldyn) in real-time.

2.4. Determination of oxidative stress markers

To obtain a 10 % tissue mixture, the right lung tissues were homogenized in a PBS buffer. The oxidative stress markers measured in this study included Reactive Oxygen Species (ROS), malonaldehyde (MDA), and reduced glutathione (GSH). ROS accumulation was quantified using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). The MDA content was determined using the malondialdehyde (MDA) assay kit (TBA method). The levels of reduced glutathione (GSH) were determined using a reagent kit from Nanjing Jiancheng Biotechnology Institute (China). All results were subsequently normalized based on the protein content of each sample.

2.5. Lung histopathology examination

To examine any potential lung abnormalities resulting from along and combined exposure, we conducted a lung histological analysis. The mice were euthanized by cervical dislocation, and the lung tissues were subsequently extracted and rinsed with phosphate-buffered saline (PBS). The left lung was utilized to prepare histopathology slices. The samples were immersed in a 4 % neutral paraformaldehyde buffer at room temperature for 24 hours, and then cut into 4-μm sections. These sections were stained with hematoxylin and eosin (H&E), and observed under an Olympus Microscope (BX53, Tokyo, Japan).

2.6. Quantitative analyses of inflammation and apoptosis

The lung tissue homogenate was centrifuged at 4°C and 10,000 rpm for 10 minutes. The resulting supernatant was collected and stored at −80°C for subsequent testing. Pro-inflammatory cytokines, tumor

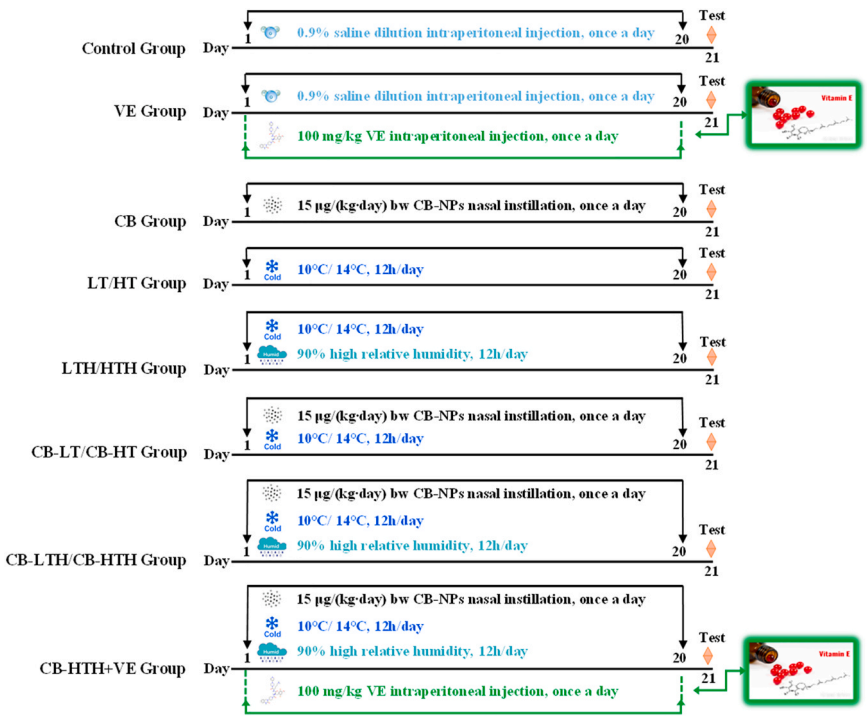

Fig. 2. Murine model establishment and exposure protocol.

necrosis factor-α (TNF-α) and Interleukin-1β (IL-1β), as well as apoptosis factors Caspase-8 and Caspase-9, were detected in the lung homogenates using ELISA kits from Shanghai FANKEL Industrial Co., Ltd. of China. The absorbance was measured at a wavelength of 450 nm using a Spectramax iD5 plate reader.

2.7. Immunohistochemical (IHC) for biomarkers

Lung tissues from mice were fixed in 4 % paraformaldehyde for 24 hours at ambient temperature. The tissues underwent the paraffin embedding process and the sections were then dewaxed and prepared for antigen retrieval. Specific antibodies, including anti-NF-κB antibody (1:400, Abcam, USA), anti-IL-6 antibody (1:200, Abcam, USA), and anti-Caspase-3 antibody (1:200, Abcam, USA), were incubated overnight at 4℃. After washing to remove the primary antibodies, HRP-conjugated secondary antibodies were applied. Visualization was achieved using the 3,3′-diaminobenzidine (DAB) chromogenic solution, and the nuclei were stained with hematoxylin. The expression intensity was captured through digital imaging and subsequently analyzed using the Image-Pro Plus 6.0 software (Media Cybernetics, USA).

2.8. Statistical analysis

GraphPad Prism 7.0 (San Diego, CA) was used to generate statistical graphs based on the experimental data. The mean ± SEM values were displayed. Data analysis was performed using SPSS Statistics V.22 Chicago, IL. To assess the significance of variations among groups, both ANOVA and Duncan's multiple range test were employed. A p-value of less than 0.05 was considered statistically significant, denoted as $p < 0.05$, $p < 0.01$.

3. Results

3.1. Decreased lung function in mice

The airway dysfunction caused by different conditions is illustrated by the airway hyperresponsiveness (AHR) measurements, as shown in Fig. 4. We observed that lower temperatures and higher humidity levels led to increased inspiratory and expiratory resistance (Ri and Re), coupled with a decrease in dynamic lung compliance (Cldyn), suggesting a pronounced airway dysfunction. The subsequent introduction of CB-NPs into this hygrothermal environment (cold-humid) intensified these effects. Intriguingly, the combination of CB-NPs with the aforementioned environmental factors caused a marked increase in Ri and Re, along with a further reduction in Cldyn. This dual impact highlights the significant role of particulate exposure in exacerbating pulmonary impairment, aligning with our previous findings (Deng et al., 2022b). However, the damage appeared to be mitigated by the administration of Vitamin E (VE), as demonstrated by the observed decrease in Ri and Re and the increase in Cldyn in VE-treated groups. This suggests VE's protective role against the deleterious effects of CB-NPs and adverse environmental conditions.

3.2. Histopathological lesions in mouse lungs

We further examined the lung histological changes resulting from exposure to a cold-humid environment and/or CB-NPs exposure. Fig. 4 illustrates the typical pathological features of airway remodeling, the control group exhibited a regular bronchial lumen without any surrounding inflammatory cell infiltration. In stark contrast, tissues exposed to the cold and humid conditions underwent remarkable airway remodeling. This was evident through pronounced wall thickening and mucosal layer folding, which was particularly intensified under combined low temperature and high humidity (Fig. 5 (LTH). The addition of carbon black particles further aggravated these histopathological changes. The CB-LTH and CB-HTH groups demonstrated severe thickening and folding of the airway walls, surpassing the alterations seen with CB-NPs or temperature and humidity stress alone (Fig. 4 - the second row). These histological markers were in alignment with the lung function impairments recorded, affirming the exacerbating role of co-exposure in pulmonary dysfunction. Moreover, the co-exposure conditions revealed a heightened response compared to single-factor exposure, correlating with noticeable changes in airway structure and function, such as airway wall wrinkling and narrowing, infiltration of

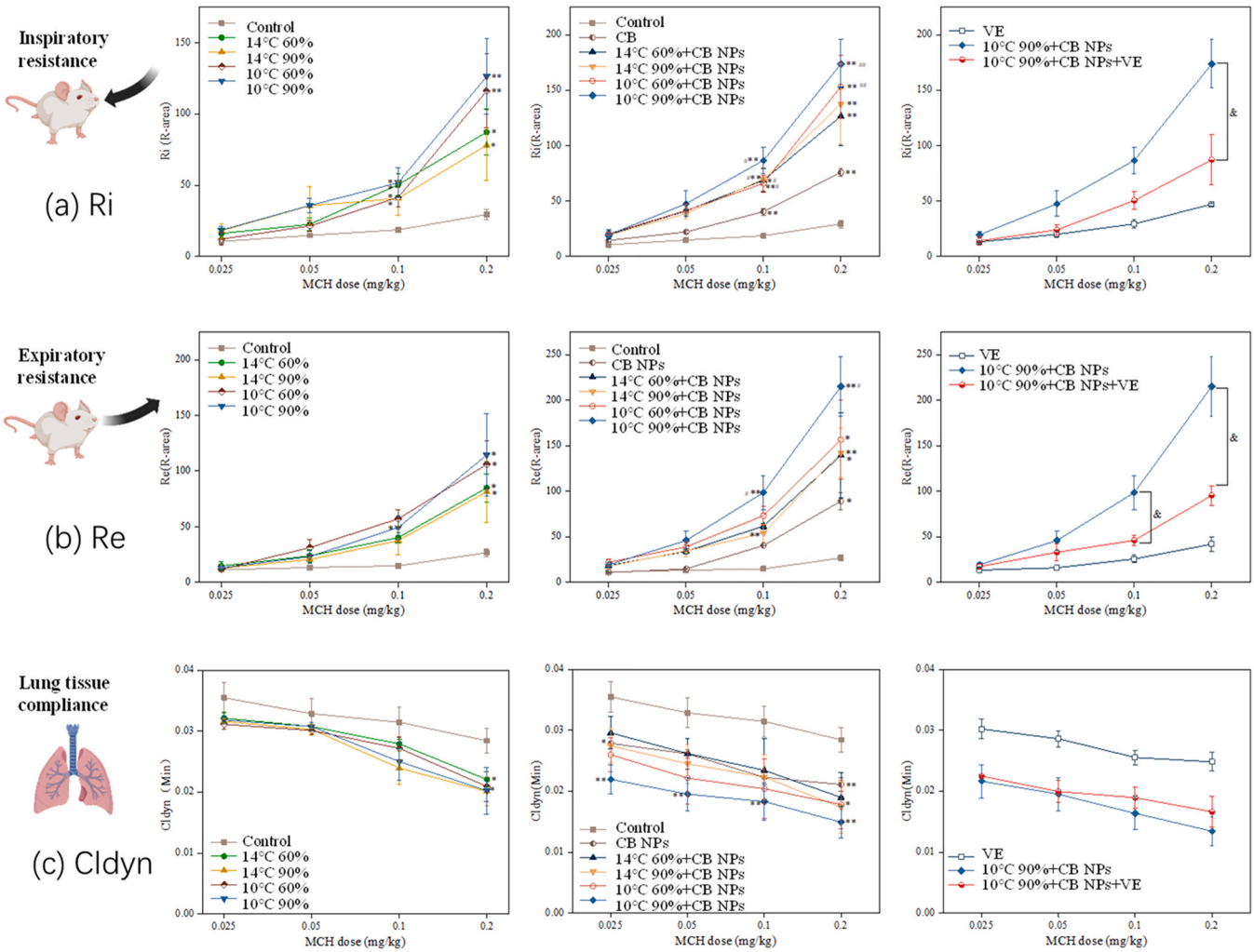

Fig. 3. Effects of exposure to environmental conditions on airway hyperresponsiveness (AHR) and amelioration by VE in mice: (a) Ri, (b) Re, and (c) Cldyn. Figures on the left are for cold-humid exposure groups (LT, HT, LTH, HTH); Figures in the middle are for different combined exposure groups: the saline control, exposure groups (CB, CB-LT, CB-HT, CB-LTH, CB-HTH); Figures on the right are comparisons between the CB-LTH group and CB-LTH + VE control group. * $p < 0.05$ and * * $p < 0.01$ (compared with control group); # $p < 0.05$ and ## $p < 0.01$ (compared with single CB exposure group); & $p < 0.05$ (corresponding conditions compared with VE group).

inflammatory cells, and an uptick in mucus secretion. This underscores the extensive damage to both large and small airways, reinforcing the validity of the observed pulmonary dysfunction. Additionally, the administration of VE showed a mitigatory effect against these pathological transformations, implying its potential as a protective agent against such environmental insults.

3.3. Oxidative damage in mouse lung tissue

Elevated levels of reactive oxygen species (ROS), glutathione (GSH), and malondialdehyde (MDA) were observed in the groups exposed to carbon black (CB) and/or the hygrothermal environments (cold-humid). In Fig. 5A it can be seen that the ROS levels significantly increased in the groups exposed to CB alone and those exposed to both the cold-humid environment combined with CB ($p < 0.01$), compared to the control group. All groups with combined exposure showed higher ROS levels compared to CB exposure alone. The two combined groups, CB-LT and CB-LTH, exhibited significant differences ($p < 0.01$). Fig. 5B shows the variations in GSH levels. There was a significant decline in GSH levels in the LTH group, CB group, and the groups with combined exposures ($p < 0.01$), compared to the control group. All combined exposure groups displayed a notable reduction in GSH levels ($p < 0.01$) compared to CB exposure alone. Although the GSH content in the HT group (14℃+ normal humidity) and the HTH group (14℃+90 % high humidity) did not differ significantly from the control group, when combined with CB-NPs, both groups displayed a significant decline. This highlights that the combination of seemingly harmless environmental factors can exacerbate negative health outcomes. Furthermore, Fig. 5C provides insights into MDA levels, confirming the trend observed in ROS. The group subjected to the combined stressors of 10℃ and 90 % humidity experienced the highest oxidative damage. This increase in MDA, indicating intensified oxidative harm to lung tissues, aligns with the observations in ROS and GSH trends. The LTH group, in particular, exhibited pronounced oxidative stress, which correlates with the abnormalities observed in airway hyperresponsiveness (AHR). These findings collectively emphasize the synergistic adverse impacts of cold and humid environments when combined with CB-NPs. Importantly, the protective potential of vitamin E (VE) was demonstrated, as its introduction to the CB-LTH+VE group significantly reduced oxidative damage levels compared to the CB-LTH group ($p < 0.01$).

3.4. Activation of transcription factor NF-κB

The activation of the transcription factor NF-κB is a crucial aspect of

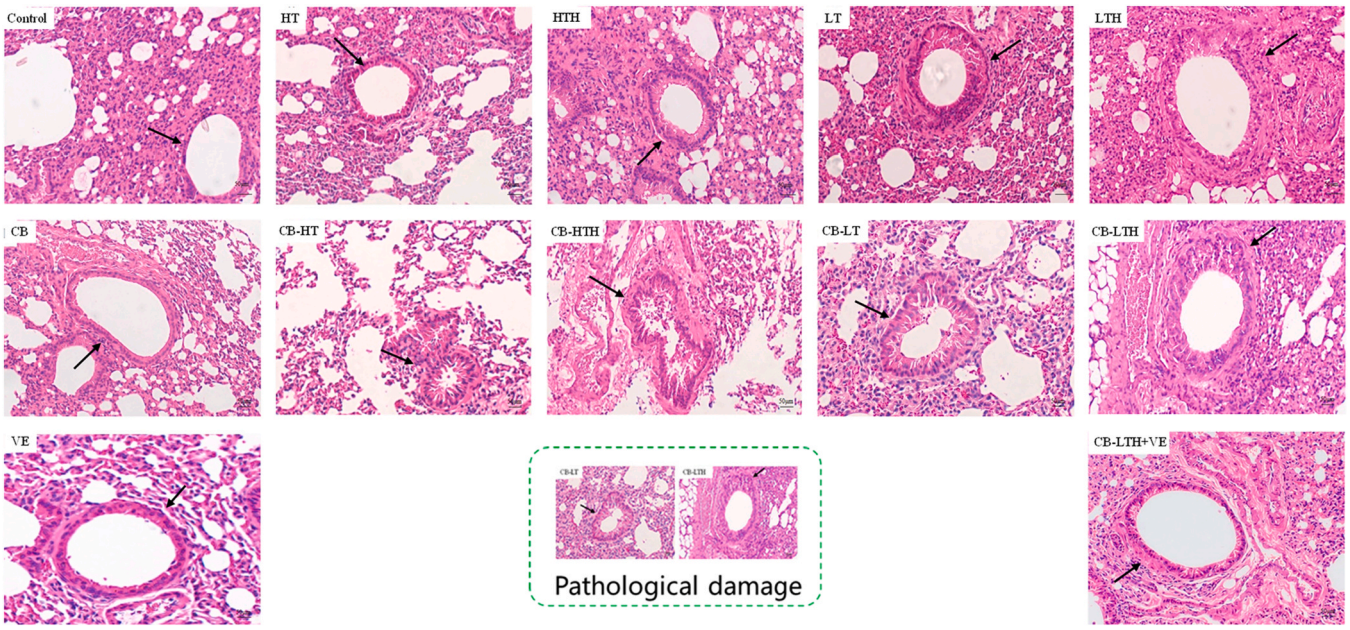

Fig. 4. Effects of exposure to different experimental groups on histopathological changes in lung tissues and quantification of lung injury: H&E stained sections revealed eosinophil proliferative inflammation and airway obstruction in lung tissue; Figures on the bottom are comparisons between the (CB-NPs-10℃+90 %) and (CB-NPs-10℃+90 %+VE) groups. The arrows indicate inflammatory cells (magnification 100x).

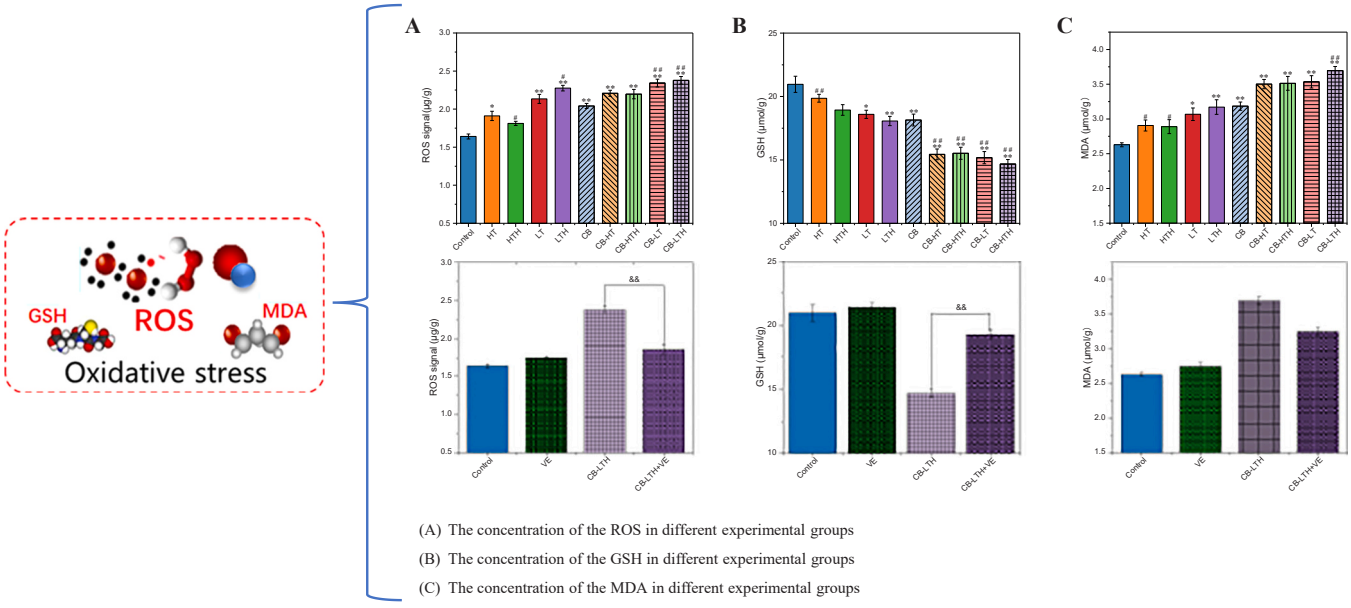

Fig. 5. Effects of the oxidative stress. CB and/or the hygrothermal environments on the levels of ROS (A), GSH (B), and MDA (C) in the lung tissues. Asterisks indicate significant differences from the control group (*p < 0.05, **p < 0.01), while hash symbols denote significant differences from the CB group (#p < 0.05, ##p < 0.01). and signifies from the CB+LTH group. (& &p < 0.01).

this study. Basal levels of NF-κB were observed in the control group, indicating a non-stimulated state (Fig. 6). Under challenging conditions with lower temperatures, NF-κB levels significantly increased, suggesting the activation of inflammatory pathways due to thermal stress. This response was further intensified when low temperatures coincided with high humidity, as indicated in the LTH and HTH groups, indicating a compounded environmental insult. Following exposure to CB-NPs, there was a significant rise in NF-κB expression, suggesting the activation of an inflammatory pathway. The complex exposure scenarios further enhanced NF-κB expression, with the CB+HTH group showing a marked increase ($p < 0.05$). Importantly, there was a statistically significant upregulation of NF-κB in the CB+LT and CB+LTH groups compared to CB-NPs alone ($p < 0.01$), indicating the synergistic effect of ultrafine carbon black particles with cold and humid environmental stressors in modulating NF-κB activity. This finding aligns with previous research documenting the role of NF-κB as an inflammatory mediator (Zhu et al., 2023). As expected, treatment with VE shows a significant down-regulation of NF-κB protein expression in the group treated with the inhibitor compared to the combined exposure group ($p < 0.01$). This indicates that VE somewhat mitigates the inflammation and apoptosis caused by the overexpression of NF-κB due to combined exposure.

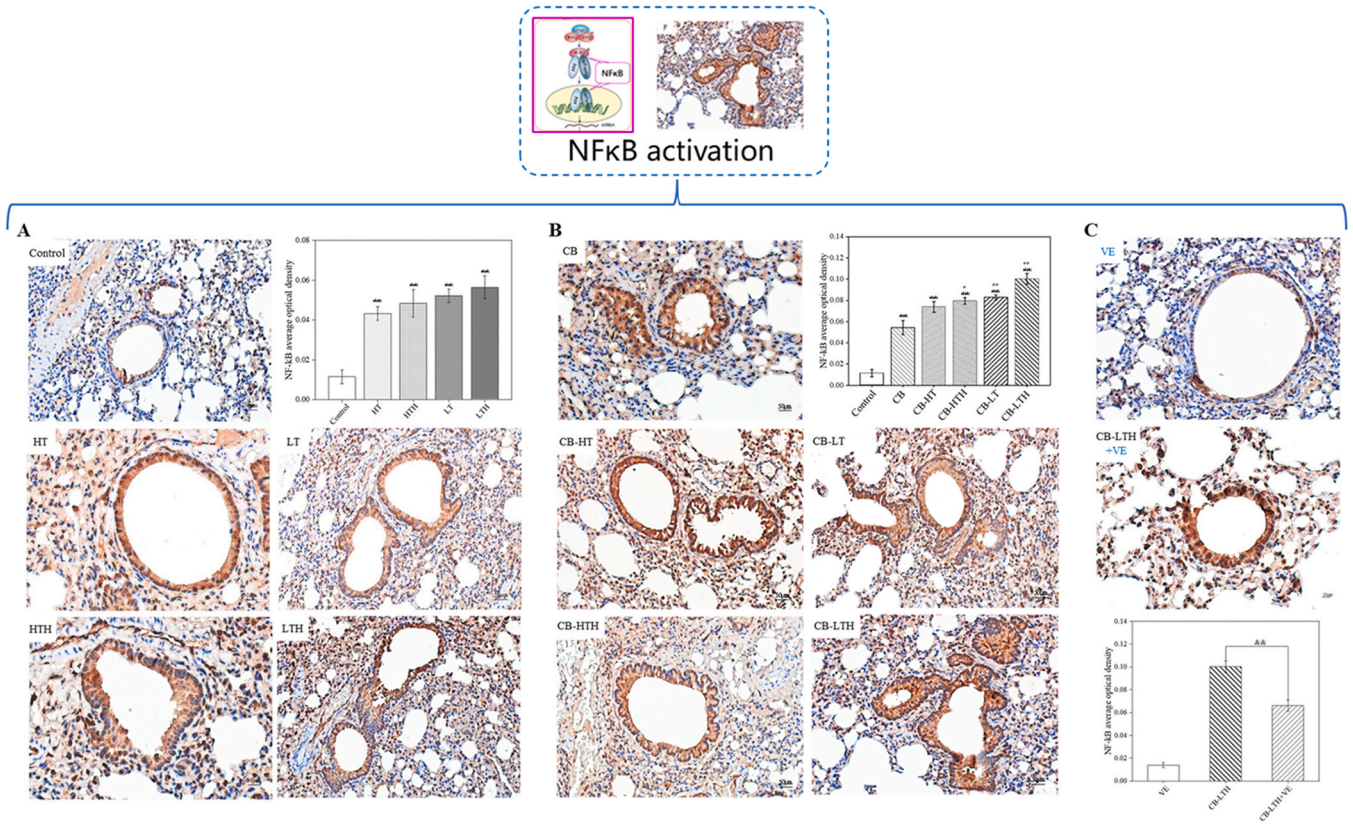

Fig. 6. Activation of transcription factor NF-κB. (A) comparison between the control group and groups that were exposed to cold or/and humidity (*p < 0.05, **p < 0.01); (B) compared with the CB group, #p < 0.05, ##p < 0.01; (C) compared with CB+LTH group, & &p < 0.01.

3.5. *Increased pro-inflammatory cytokines*

Fig. 7 illustrates the impact on pro-inflammatory cytokine levels. Fig. 7(A) reveals that groups subjected to combined exposures of cold-humid environment and CB display significantly heightened IL-6 levels, indicative of an escalated immune response. This finding is in

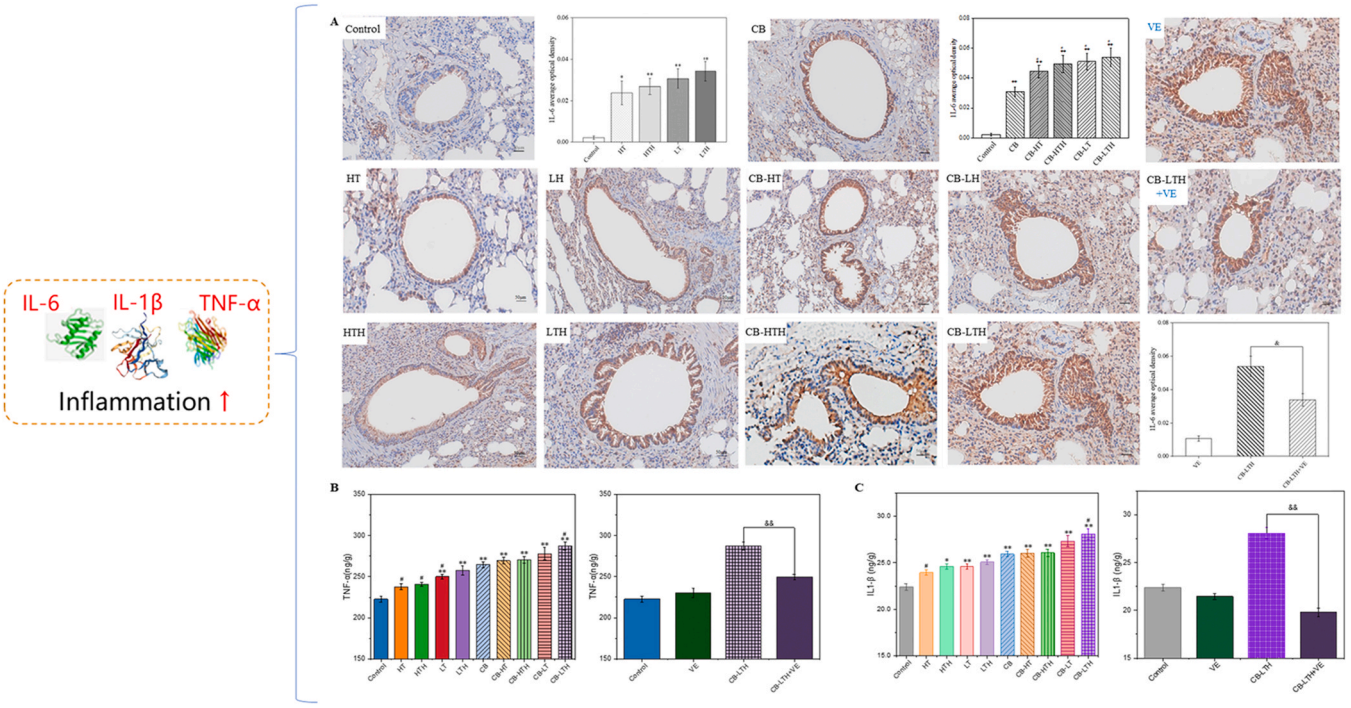

Fig. 7. The levels of the pro-inflammatory cytokine in different experimental groups (*p < 0.05, ** p < 0.01, compared with the control group; compared with the CB group; # p < 0.05, ## p < 0.01; compared with CB+LTH group, & p < 0.05, & &p < 0.01).

line with the role of IL-6 in regulating immune responses during airway inflammation (Aliyu et al., 2022). As shown in Fig. 7(B), a marked increase in TNF-α levels is observed in most groups compared to the control group ($p < 0.01$), with the exception of the HT and HTH groups. The CB-LTH group, exposed to 10°C and 90 % humidity, showed the most significant rise, pointing to a strong synergistic effect ($p < 0.01$). According to Fig. 7(C), IL-1β levels varied across groups. The HT group showed no significant change compared to the control group. However, the HTH group experienced a considerable increase ($p < 0.05$). The 10°C groups, both at 60 % and 90 % humidity, demonstrated a highly significant elevation in IL-1β levels ($p < 0.01$). VE offered effective protection with a noticeable decrease in the expression levels of IL-6 ($p < 0.05$) and a significant reduction in the pro-inflammatory cytokines TNF-α and IL-1β ($p < 0.01$) when compared to the CB-LTH group.

3.6. Analysis for apoptotic biomarkers

Fig. 8 elucidates the expression of apoptotic biomarkers. Caspase-3, an essential indicator of apoptosis, showed heightened expression across all exposure groups relative to the control group (Fig. 8A). Compared to CB alone, the 14°C co-exposure groups did not exhibit a statistically significant increased response. Conversely, in the 10°C co-exposure groups (CB-LT and CB-LTH) the response was markedly pronounced ($p < 0.05$ for CB-LT and $p < 0.01$ for CB-LTH, respectively). This indicates a temperature-dependent sensitivity in the apoptotic response, highlighting the unique influence of lower temperature conditions on apoptotic mechanisms (K. E. King, J. J. McCormick, and G. P. KennyAdvanced Biology). Fig. 8B and Fig. 8C reveal variations in the levels of Caspase-8 and Caspase-9, both of which are key initiators in the apoptotic pathway. These markers showed an increase across all groups subjected to co-exposure compared to control. Notably, compared to CB alone, under moderate cold stress conditions (14°C), there was only a moderate rise in these markers, without significant statistical differences. However, under severe cold stress conditions (10°C), with either normal or high humidity, there was a significant escalation in Caspase-8 and Caspase-9 levels ($p < 0.05$ and $p < 0.01$, respectively), underscoring the heightened impact of extreme cold stress on apoptosis initiation. As anticipated, the use of Vitamin E led to a considerable reduction in the levels of Caspase-8, Caspase-9, and Caspase-3 in the VE-treated group compared to the CB-LTH group, with the differences being statistically significant ($p < 0.01$).

4. Discussion

The Effect of Combined Exposure in Exacerbating Lung Injury: The mechanism behind combined exposure is delineated in the following steps: (1) A 21-day exposure to a cold and humid environment in conjunction with CB-NPs leads to elevated levels of reactive oxygen species (ROS) in the lung tissues of mice. As the CB-NPs are absorbed through the respiratory system and reach the lung cells (Nho, 2020), they contribute to an increase in intracellular ROS levels during metabolism. (2) The rise in ROS levels induces cellular oxidative stress, characterized by a reduction in glutathione (GSH) concentration and an increase in malondialdehyde (MDA) levels (Juan et al., 2021). (3) Oxidative stress triggers the activation of transcription factors, inflammatory responses, and apoptosis (Araujo, 2011; Chatterjee, 2016). This can stimulate the synthesis and release of inflammatory factors such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) through the NFκB signaling pathway, leading to tissue inflammation. TNF-α can also induce apoptosis, particularly affecting lung tissue (Malaviya et al., 2017). (4) The cascade of oxidative stress, inflammatory responses, and apoptosis ultimately leads to a deterioration in lung function (Chaudhary et al., 2023). Experimental models induced with VE treatment may demonstrate that oxidative stress and inflammatory responses leading to cellular apoptosis in lung tissue result in pulmonary dysfunction. The interplay between oxidative stress and apoptosis may intensify each other, with a significant increase in apoptotic biomarkers, including Caspase-3, Caspase-8, and Caspase-9, highlighting the extent of cellular death. (5) Vitamin E acts as a preventative and therapeutic agent. By scavenging ROS molecules in tissues and cells, it inhibits the rise in ROS levels, thereby addressing the initial event in the pathological molecular mechanism. Therefore, Vitamin E has the potential to prevent and treat the effects of combined exposure in mouse models.

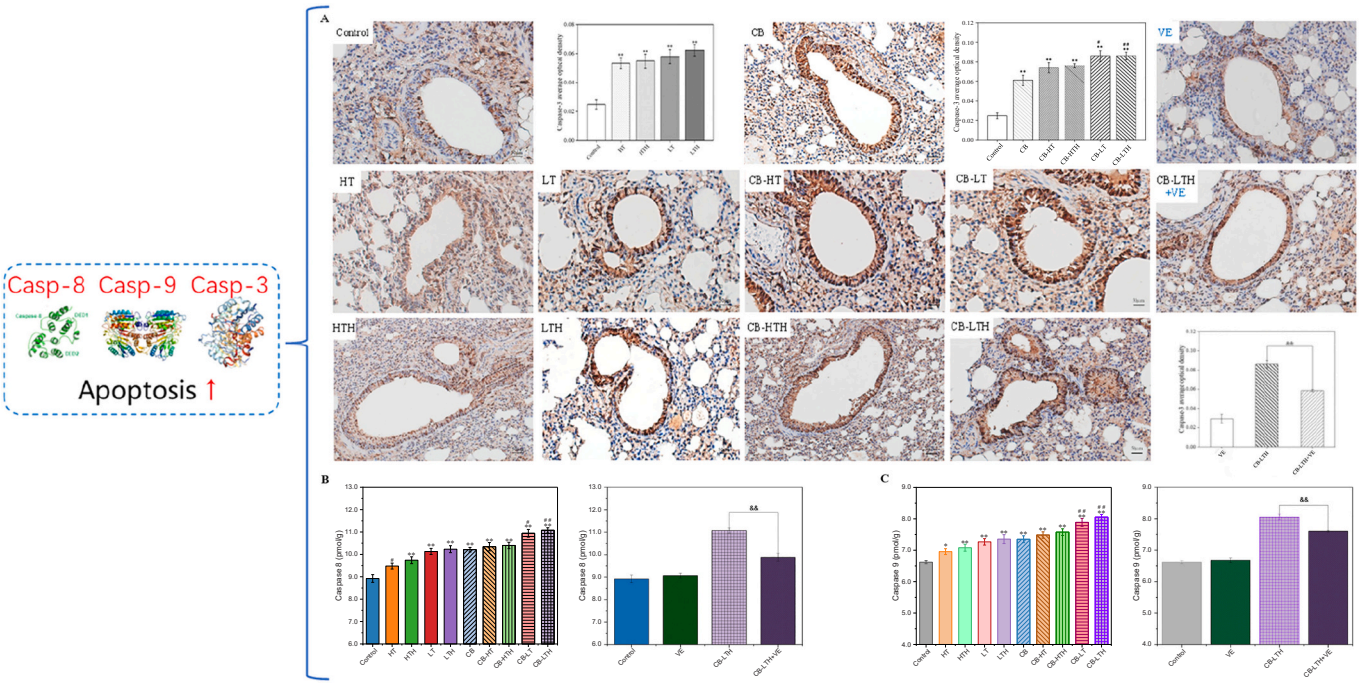

Fig. 8. The concentration of the biomarker in different experimental groups (*$p < 0.05$, ** $p < 0.01$, compared with the control group; compared with the CB group; # $p < 0.05$, ## $p < 0.01$; compared with CB+LTH group, & $p < 0.05$, & &$p < 0.01$).

4.1. Evidence that Cold and Humid Environments Worsen Lung Function

Single exposures to low temperature, cold-humid conditions, and CB-NPs each led to a decline in lung function and exacerbated pathological changes. This included airway remodeling in mice, triggering increased oxidative damage, inflammatory responses, and apoptosis, culminating in lung injury. Previous studies have well-documented the lung injury caused by CB-NPs (Madl et al., 2014). Notably, our study also focuses on the impacts of temperature and humidity. Under cold experimental conditions, we observed a significant increase in lung damage, which was further intensified by an increase in humidity at the same temperature level, with the LTH experimental group (10°C + 90 %) experiencing the most severe adverse effects. In a humid environment, the moisture carried by cold air condenses rapidly within the airways upon inhalation, not only causing airway smooth muscle contraction but also leading to airway mucosal dryness and stimulating an increase in mucus gland activity, thereby exacerbating mucus secretion (D'Amato et al., 2018a; Koskela, 2007). Moreover, this process might weaken the airway's local immune defense mechanisms, making the respiratory tract more susceptible to infections and triggering inflammatory responses (D'Amato et al., 2018b; Guarnieri et al., 2023). Concurrently, we propose that cold and humid environmental conditions increase oxidative stress, leading to the generation of free radicals that cause damage to cells and tissue structures, thereby further exacerbating lung tissue damage.

4.2. Evidence that Combined Exposure Exacerbates Lung Function

The detrimental impacts of climate change on the distribution and toxicity of environmental pollutants are becoming increasingly apparent (Noyes et al., 2009). However, in these studies, temperature and humidity are often incorporated as confounding factors in the analysis (Zanobetti and Peters, 2015), hence limiting the understanding of the comprehensive impact of pollution on health. The intersection of evolving climate conditions with pollution exposure poses significant challenges to public health, particularly through the exacerbation of cold, humid conditions, and particulate matter pollution (Klompmaker et al., 2021; Wu et al., 2022; Tian et al., 2020; Wine et al., 2022). These factors collectively contribute to an increased risk of hospitalization and morbidity associated with cardiovascular and respiratory diseases. Vulnerable populations, including the elderly, infants, children, and those with pre-existing chronic cardiopulmonary and immune diseases, are at heightened risk of suffering from these compounded environmental stressors (Lepeule et al., 2018b). Given the potential severity of these synergistic interactions, direct investigation into the toxicological mechanisms under climate change and pollutant exposure is necessary. Our study found that temperature and humidity have a synergistic effect on lung injury when combined with ultrafine particulate matter exposure, compared to exposure to CB-NPs alone. Notably, our experiments included two low temperatures, 14℃ and 10℃. Despite the minor temperature difference, CB-NPs showed no significant impact in combination with 14℃, whereas significant changes in indicators were observed at 10℃. Moreover, an increase in humidity (90 %) amplified the damaging effects, highlighting the dangers of synergistic effects. While some studies suggest that temperature and humidity conditions directly change the deposition of aerosols in the respiratory tract, thereby affecting lung function (Xi et al., 2013; Xu et al., 2021), our experiments introduce a new discovery: from a toxicological perspective, inhaling cold, humid air independently impacts lung function and reduces immunity levels, thereby exacerbating pollution from ultrafine particulate matter to the lungs. Our observations emphasize the complex risks associated with environmental exposure, particularly, the finding that low temperature and high humidity enhance the impact of particulate matter pollution.

4.3. Dual Insights from Vitamin E Intervention

Vitamin E is regarded as an effective antioxidant that has been proven to efficiently neutralize and quench reactive oxygen species (ROS), underscoring its vital role in alleviating oxidative stress (Liao et al., 2022; Rizvi et al., 2014). Given the manifestation of oxidative stress and inflammation in lung damage, the antioxidative and anti-inflammatory properties of Vitamin E are particularly critical. In our study, after intranasal instillation of ultrafine particulate matter, an intraperitoneal dose of 100 mg/kg/day of Vitamin E was administered to the experimental animals, unveiling two significant findings regarding Vitamin E as a signaling pathway blocker: Firstly, the use of Vitamin E supported the hypothesis that elevated ROS levels act as an upstream initiating event in the mechanism of lung damage. Secondly, the application of Vitamin E to the group most severely damaged by combined exposure (CB-LTH+VE group) showed significant improvements in inflammatory and apoptosis biomarkers ($p < 0.001$), thereby reinforcing the notion that oxidative stress serves as a core molecular mechanism by which combined exposure to air pollutants exacerbates lung damage in mice. The dosage of Vitamin E used in this study (100–200 mg/kg) aligns with the dosage range applied in previous studies addressing oxidative stress induced by particulate pollution. Our experimental results further highlight the potent protective effect of an adequate amount of Vitamin E on lung damage in balb/c mice, indicating its potential as a preventive and therapeutic strategy against the effects of environmental combined exposure.

4.4. Limitations of the Study

It is imperative to acknowledge the principal limitations of our work. The 21-day exposure period illuminated the synergistic effects of combined exposure. While this duration has successfully demonstrated the acute effects of environmental stressors, the typical characteristics of health effects induced by pollutant toxicity under changing physical environments include low dosage, long-term exposure, and cumulative characteristics (Manisalidis et al., 2020; Pope, 2000). Thus, extending the duration of exposure in future research will likely provide further insights into chronic health outcomes.

5. Conclusions

Through molecular toxicological research, this study explores the correlation and potential molecular pathomechanisms between lung injury and combined exposure to cold-humid environments and ultrafine particulate matter ($PM_{1.0}$). We observed that both cold humid environments and ultrafine particulates independently induce significant alterations in lung tissue. These alterations are characterized by an exacerbated response in oxidative stress markers, inflammatory cytokines, and apoptotic indicators within the pulmonary cells of mice. Notably, the adverse effects of ultrafine particulate exposure are significantly amplified under cold and humid conditions, suggesting a synergistic exacerbation of lung injury mediated by the NF-κB signaling pathway and augmented cellular oxidative stress. Remarkably, the intervention with Vitamin E markedly mitigated these adverse effects, underscoring its substantial protective role against lung damage from the detrimental impacts of environmental stressors. Crucially, our research underscores the necessity of integrating considerations of climatic factors (temperature, humidity, and particulate matter exposure) in the conceptualization and design of indoor environments. This is essential for safeguarding respiratory health, particularly in settings prone to fluctuating environmental conditions. The implementation of our findings into indoor environment design involves the development of strategies aimed at mitigating the combined effects of these stressors. Such strategies could include enhancing ventilation, employing air purification systems to reduce particulate matter levels, and maintaining optimal humidity and temperature levels to prevent exacerbation of

particulate matter toxicity. By adopting these approaches, we can significantly improve indoor air quality thereby promoting healthier and safer living spaces.

CRediT authorship contribution statement

Lixiang Wu: Visualization, Formal analysis. **Ziyu Shu:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Runming Professor Runming:** Writing – review & editing, Funding acquisition, Conceptualization. **Ping Ma:** Validation, Project administration, Methodology. **Yang Wu:** Resources, Methodology. **Shuo Qing:** Writing – original draft, Methodology, Investigation, Data curation. **Xu Yang:** Writing – review & editing, Visualization, Supervision, Methodology, Conceptualization. **Baizhan Li:** Supervision, Project administration, Funding acquisition. **Fangxin Fang:** Writing – review & editing, Validation, Supervision.

Declaration of Competing Interest

The Authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.117638.

Data availability

Data will be made available on request.

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