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Non-inflammatory emphysema induced by NO_2 chronic exposure and intervention with demethylation 5-Azacytidine

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ABSTRACT

Aims: A rat model of emphysema was established that mimics the features of the human emphysema subtype and explores the effects of demethylation on lung function and blood tests.

Materials and methods: Rats were randomly assigned to NO_2, NO_2 + 5-Azacytidine, and normal air groups based on a emphysema rat model induced by chronic NO_2 exposure. This study estimates the characteristics of emphysema by conducting an analysis for IL-6 and TNF-α levels in bronchoalveolar lavage fluids (BALF) and plasma. Furthermore, CD68 macrophage immunofluorescent staining and inflammatory cell counts in BALF were compared between rats exposed to NO_2 and normal air.

Key findings: 5-Azacytidine treatment led to restored Δweight at 14 and 75 days of intervention and NO_2 + 5-Azacytidine significantly reversed the effect of NO_2 exposure on Δweight. Intervention with 5-Azacytidine alleviated the decline of pulmonary function with a significant increase in FEV100/FVC% at 75 days in NO_2 + 5-Azacytidine rats compared to NO_2 rats. 5-Azacytidine reduced the counts of white blood cells (WBCs), granulocytes, lymphocytes, and monocytes at 14 days, but increased WBC, granulocyte, and monocyte counts at 45 days. Red blood cell counts, hemoglobin, and hematocrit concentrations were significantly reduced in NO_2 + 5-Azacytidine rats.

Significance: This non-inflammatory rat emphysema model (induced by chronic NO_2 exposure with global DNA hypomethylation and demethylation therapy with 5-Azacytidine) effectively improved emphysema by alleviating the decline of lung function and hypoxia, and slightly reinforced immune function. These results indicate the therapeutic potential of demethylation agents for the prevention and treatment of emphysema induced by the air pollutant NO_2.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive disease of the lungs, which is characterized by a (not fully reversible) limitation of the expiratory airflow, chronic inflammation, obstruction of airways, and emphysematous destruction of lung tissue. Pathologically, COPD is characterized by three interrelated processes: 1) the remodeling of small airway walls, the loss of small airways, and emphysema; 2) the destruction of the alveolar structure leading to airspace enlargement and the loss of elastic recoil; and finally 3) the loss of peribronchiolar attachments. Although smoking plays an important role in COPD, evidence suggests that this type of disease could also have other etiologies [1].

Air pollutants have the ability to contribute to the development of COPD [2]. Exploration of one or two specific pollutants with the greatest contribution to the detrimental health effects of COPD could have pivotal implications for environmental and social policies, as well for the local government toward the protection of public health. As a

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major anthropogenic emission, NO_2 is a traffic-sourced air pollutant with severe health effects [3]. Due to its broad diffusion and strong oxidization, NO_2 has drawn wide attention from public health professionals and policy makers. Earlier work demonstrated the adverse effects of NO_2 on human health [4], especially for COPD [5]. Particularly, our latest meta-analysis identified consistent evidence for a potential association between NO_2 and COPD in adults (Zhang et al., 2018). However, further studies on possible mechanisms are not available.

Animal models are of great importance for the study and advancement of knowledge on the determinants of pathological processes of COPD. Therefore, experiments were designed in which rats were chronically exposed to 20 ppm of NO_2 to gain understanding about the mechanism of NO_2 action in the development of COPD. These experiments were novel since they offer a relatively new phenotype model of COPD. According to a recent meta-analysis, similar models of COPD were induced by cigarette smoke extract (CSE) exposure, intra-tracheal lipopolysaccharide (LPS), and intranasal elastase; only variations in time span and dose of inducers have been employed in different studies [6].

DNA-methylation, as one of the epigenetic regulators, is a pivotal element for normal lung function, and studies have identified DNA-methylation as significantly related to COPD susceptibility and severity. Recent studies have also associated underlying significant CpG loci with genetic and epigenetic pathways that could have the ability to contribute to COPD development [7,8]. Accumulating evidence indicates that epigenetic changes could be affected by air pollution. In particular, studies showed global DNA hypomethylation and DNA hypermethylation at specific functional regions in genes associated with air pollutants [9–11]; however, such effects excusive to the air pollutant NO_2 have not been studied to date. This study investigated whether differences in global DNA methylation could be induced by chronic exposure to NO_2. DNA hypermethylation in specific genes plays central roles in disease development. However, previous studies did not report the underlying mechanism on the mitigation of COPD by DNA demethylation drugs.

Therefore, this study further estimated the characteristics of an emphysema rat model induced by chronic NO_2 exposure. The aim was to explore the effects of NO_2 on global DNA methylation and the effects of demethylation on lung function and blood cells. The results provide direction for future studies on the functional genes that are specially influenced by NO_2 exposure and associated with disease.

2. Methods

2.1. Study design

For each experimental time point (i.e. the 14th, 45th, and 75th day after start of exposure), rats were anesthetized using Pentobarbital (50 mg/kg body weight) and pulmonary function was measured at each given time. Immediately upon the onset of complete apnea, blood was drawn via cardiac puncture into vacuum containers containing EDTA as previously described [12]; peripheral blood was used for further research. Three groups of rats were investigated at each time endpoint (14th, 45th, 75th day of exposure) and exposed to either NO_2, NO_2 + 5-Azacytidine, or normal air. Rats received intraperitoneal injection of 5-Azacytidine (EY0611, SIGMA, USA) at 10 mg/kg/day [13]; 5-Azacytidine is a methyltransferase inhibitor that leads to DNA demethylation and was dissolved in 0.85% NaCl solution immediately prior to use due to its unstable nature.

2.2. NO_2 exposure chamber and animals

Detailed information about the NO_2 exposure system is presented in the Supplementary material. Rats were exposed to NO_2, NO_2 + 5-Azacytidine, or normal air for 14, 45, and 75 days; n = 4 or 5 for 14 and 45 days of exposure except for the 75 d group where n = 5 or 6 (no more than four rats per cage). Experiments used male Sprague-Dawley (SD) rats with an initial body weight of 180–200 g, obtained from Southern Medical University (Guangzhou, China) and managed at the Central Animal Facility of Guangzhou Medical University. The rats were continuously exposed to the treatments (from 8 a.m. until 2 p.m.) for six consecutive days per week for different periods. The NO_2 + 5-Azacytidine rats, which were injected with 5-Azacytidine, were managed identically to the NO_2 exposed rats and were housed in similar cages. During exposure, rats could freely move and had access to water and food. During resting periods, all rats breathed normal air. The study procedures and protocols were approved by the institutional review boards of Guangzhou Medical University and were conducted in accordance with the institutional guidelines on the Care and Use of Laboratory Animals (National Institutes of Health).

2.3. Physiological testing of pulmonary function

Spirometry was performed using a forced pulmonary maneuver system following the manufacturer's protocol (Buxco Research Systems, Wilmington, North Carolina, USA). These procedures have been previously described in detail [14]. The Total Lung Capacity (TLC), Forced Vital Capacity (FVC), and Chord Compliance (Cchord) were measured. With the fast flow volume maneuver, the Forced Expiratory Volume in 1 ms (FEV100) and the FVC were recorded from the best of three measurements.

2.4. Immunofluorescent staining

The first resultant slide (4 μm thickness) from each case was subjected to immunofluorescent analysis. Tissue sections were dewaxed and rehydrated in both ethanol and water. After the antigen was retrieved, the sections were washed thrice in phosphate-buffered saline (PBS). Nonspecific binding was blocked with 5% goat serum (CWBIO, Beijing, China). The sections were incubated overnight at 4 °C with the primary antibodies of CD68, washed thrice with PBS, and incubated at room temperature with secondary antibodies AlexaFluor 488-conjugated AffiniPure goat anti-rabbit secondary Ab1:200 (Proteintech Group, Inc., USA) for 1 h. After the sections were washed, the cell nuclei were counterstained with fluorescent mounting medium containing 4, 6-diamidino-2-phenylindole (Abcam, Cambridge, UK).

2.5. Hematological tests

Peripheral venous blood samples were drawn after each predetermined time and hematological parameter such as Hemoglobin (HGB), Hematocrit (HCT), red blood cell (RBC) items, and total and differential leukocyte counts were determined using the animal exclusive Automated Hematology analyzer (Mindray, BC-2800). Differential leukocyte counts (as percentages) were multiplied by the total white blood cell counts and were expressed as concentrations of cells. The remainder of the samples was centrifuged and aliquots of plasma were kept at −80 °C for a series of biochemical tests. High-Density Lipoprotein Cholesterol (HDL-C), Low-Density Lipoprotein Cholesterol (LDL-C), Glucose (GLU), total cholesterol (TC), and Triglyceride (TG) levels were measured by using an auto-analyzer (Chemray 240, RaytoInc, Shenzhen, China). The content of serum Glycated Serum Protein (GSP) of rats was measured via colorimetry (UV–visible spectrophotometer 530 nm).

2.6. Bisulfite-modified methylation-specific PCR (MS-PCR)

DNA methylation occurs via covalent addition of a methyl group at the 5-carbon of the cytosine ring, which results in 5-methylcytosine. Bisulfite modification of genomic DNA was followed by PCR amplification, cloning, and sequencing of individual PCR amplimers and yielded reliable information on the methylation states of individual cytosines on individual DNA molecules. By treating DNA with bisulfite

(Epigentek, A-P-1026), cytosine residues were deaminated to uracil while leaving 5-methylcytosine intact. The Methylamp™ MS-qPCR Fast Kit (Epigentek, A-P-1028) provided a master mix format, containing a hot start DNA polymerase, dNTPs, an MS-PCR enhancer, an optimized buffer, and an intercalating green dye. This master mix enables a convenient and simple reaction setup. The unique hot start DNA polymerase is provided in an inactive state at ambient temperature and is reactivated via several minutes of incubation at 95 °C, which can easily be integrated into required thermal cycling steps. The hot start DNA polymerase in combination with the optimized buffer ensures MS-qPCR specificity and sensitivity. The green dye enables DNA detection and analysis without the requirement for a sequence-specific probe.

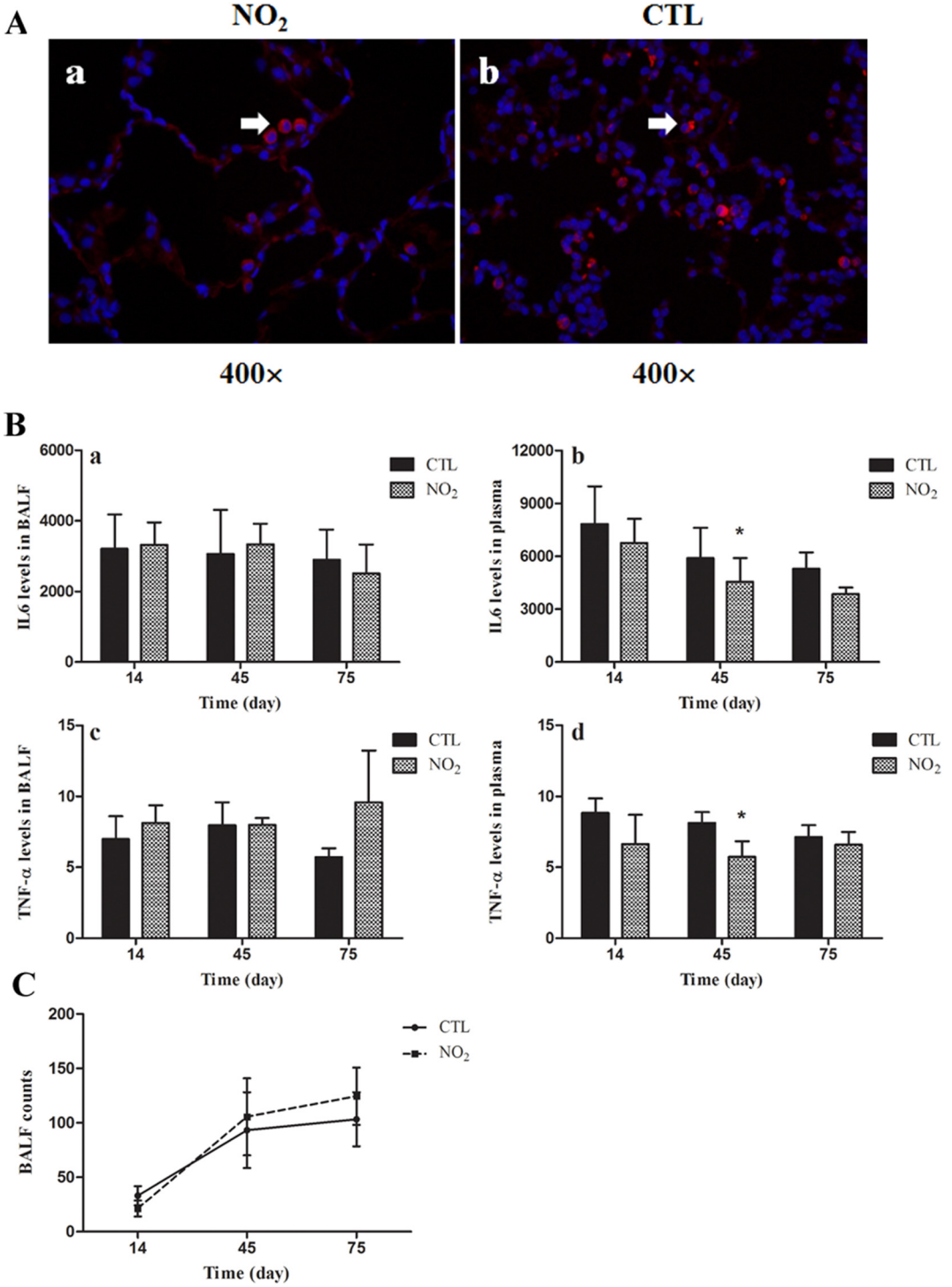

Fig. 1. (A) Immunofluorescent staining of CD68 in NO_2 exposed rats and the corresponding normal air controls after 75 days of exposure. The density of macrophages was calculated by counting the number of CD68-positive cells in every high-power field. CD68 stained in red was used as marker for macrophages from lung tissue, while DAPI, stained in dark blue, was used as a marker for cell nuclei (400 ×). (B) Histograms (a–d) showing cytokine levels (IL-6 and TNF-α) of BALF and plasma in rats exposed to NO_2 or normal air at different time points (14, 45, and 75 days). For NO_2 exposure (gray color) vs normal air (black color), $p < 0.05$. Line graph C shows the inflammatory cell counts in BALF in rats exposed to NO_2 or normal air at different time points (14, 45, and 75 days). For NO_2 exposure (solid line) vs normal air (dotted line), $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.7. Inflammatory cell counts in bronchoalveolar lavage (BAL) fluids

BAL fluid was obtained by BAL performed once with 5 mL sterile saline. The recovered lavage fluid was centrifuged at 5000 rpm for 5 min. The cell free supernatants were collected and stored at −80 °C until further analysis.

2.8. Cytokine in BALF and plasma

BALF and plasma levels of IL-6 and TNF-α in rats were measured via ELISA according to the manufacturer's instructions (Rat IL-6 ELISA kit: 900-M86, Peprotech; Rat TNF-α ELISA kit: KRC3011, Peprotech).

2.9. Statistical analyses

In general, data of quantitative traits were described as means and standard deviations (means ± SD). When data were not normally distributed, both median and inter-quartile range (IQR) were employed to describe variables. At each given time (14, 45, and 75 days of treatment), pulmonary function, blood tests, and Δweight were compared between NO_2 and NO_2 + 5-Azacytidine groups or between NO_2 and normal air groups using *t*-test (or). At each given time (45, 75, and 105 days of treatment), the global DNA hypomethylation between NO_2 group and normal air comparisons were performed using *t*-test. At 105 days of treatment, the global DNA methylation, IL6, and TNF-α among NO_2, NO_2 + 5-Azacytidine, and normal air group were performed using one-way ANOVA analyses with Bonferroni correction. All analyses were conducted with GraphPad Prism 5 software, using two-sided *p* values. Statistical significance was assumed at $p < 0.05$.

3. Results

3.1. CD68 macrophage immunofluorescent staining

Immunofluorescent staining was used to investigate both the distribution and phenotype of macrophages in lung tissues. At 75 days in the NO_2 group, when emphysema had successfully been induced (Fig. S1), CD68 macrophage infiltration showed no statistically significant increase between NO_2 group and normal air group as indicated by immunofluorescence results shown in Fig. 1A and Fig. S2.

3.2. Inflammation cytokine assay of IL-6 and TNF-α

The levels of IL-6 and TNF-α in BALF and plasma were evaluated. After 45 days of NO_2 inhalation, IL-6 showed a slight but statistically significant increase compared to control in plasma ($p < 0.05$). Similarly, after 45 days of NO_2 exposure, TNF-α level showed a weak but significant increase compared to control in plasma ($p < 0.05$); however, no statistically significant increase was found after 75 days of exposure (Fig. 1B).

3.3. Inflammatory cell count in BALF

Inflammatory cell counts in BALF were also measured at each time point. At 75 days of NO_2 inhalation, when emphysema had successfully been induced, inflammatory cell counts in BALF of the NO_2 group were not statistically significant different compared to control ($p > 0.05$ for all; Fig. 1C). In addition, for peripheral venous blood cells (counts of WBC, granulocytes, lymphocytes, and monocytes), the same tendency was found (Fig. S3).

3.4. Effects of NO_2 exposure on flobal methylation

Global methylation levels were tested at different time points. As shown in Fig. 2, the global methylation levels of genomic DNA were significantly decreased in rats exposed to NO_2 compared to normal air group both after 45 days and 75 days of exposure ($p < 0.05$, for all). Especially, one month after exposure (105 days) the significant difference was still found ($p < 0.05$).

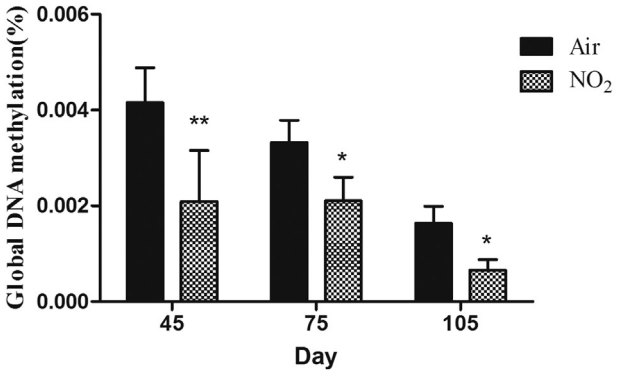

Fig. 2. Histogram showing global DNA methylation levels at different time points (45 and 75 days) of exposure to NO_2 or normal air, as well after one month of recovery with exposure to normal air (at 105 days). For NO_2 exposure (fine grid) vs normal air (black color), $p < 0.05$.

3.5. Effects of 5-Azacytidine on pulmonary function and on global methylation level

Dynamic spirometry was evaluated at each time point. After 45 days of NO_2 + 5-Azacytidine exposure, FEV100/FVC% showed a statistically significant decrease compared to the NO_2 group ($p < 0.05$). This decrease was reversed and an improving effect of 5-Azacytidine on FEV100/FVC% was found after 75 days of exposure (see Fig. 3). Treatment with 5-Azacytidine unexpectedly alleviated the decline of pulmonary function. Compared with normal air group, NO_2 + 5-Azacytidine could have the ability to decrease the global methylation level ($p < 0.05$), and there was no statistically significant difference between NO_2 and NO_2 + 5-Azacytidine ($p > 0.05$) in Fig. S4.

3.6. Effects of 5-Azacytidine on ratio of different organs and Δweight changes

The ratios of different organs (heart, liver, spleen, and kidney) to their own weight and Δweight in rats were compared between NO_2 + 5-Azacytidine and NO_2 groups at different given time points. Compared to the NO_2 group, treatment with 5-Azacytidine resulted in restored Δweight at 14 and 75 days of NO_2 exposure in Fig. 4 (because the severe Δweight reduction caused by NO_2 inhalation compared to normal air group Fig. S5); comparisons between NO_2 + 5-Azacytidine and NO_2 showed that treatment with 5-Azacytidine resulted in an increased liver to body weight ratio ($p < 0.05$) as shown in Fig. 4.

3.7. Effect of 5-Azacytidine on blood leukocyte concentrations and biochemical test

Determination of RBC, HGB, and HCT concentrations showed reduced levels in the NO_2 + 5-Azacytidine group ($p < 0.05$ for all comparisons at different NO_2 exposure times as shown in Fig. 5). At 14 days, WBC, granulocyte, lymphocyte, and monocyte counts decreased in the NO_2 + 5-Azacytidine group, and the same pattern was also found at the 75 days end point ($p < 0.05$). Administration of 5-Azacytidine did not affect the levels of GLU, GSP, HDL-C, LDL-C, TG, and TC in the NO_2 + 5-Azacytidine group compared to the NO_2 group ($p > 0.05$; see Fig. 6.

3.8. Effect of 5-Azacytidine on inflammation cytokine IL-6 and TNF-α

Levels of IL-6 and TNF-α in BALF and plasma were evaluated at

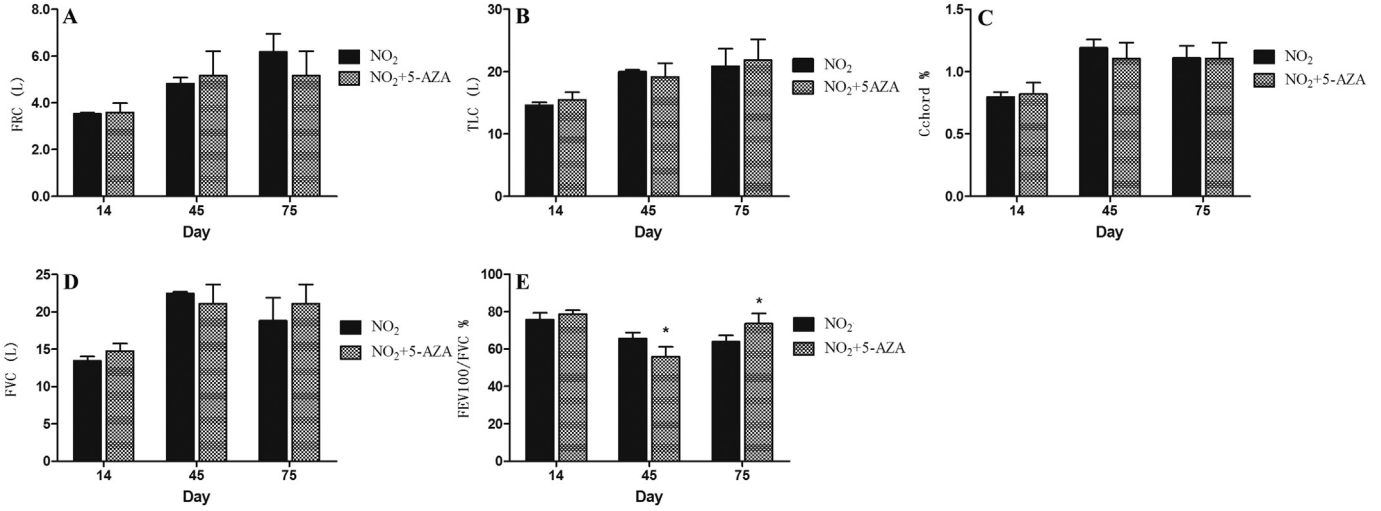

Fig. 3. Histograms (A-E) show pulmonary function index (FRC, TLC, Cchord%, FVC, and FEV100/FVC%) at different time points (14, 45, and 75 days) in the NO_2 group compared to the NO_2 + 5-Azacytidine group. For NO_2 exposure (black color) vs NO_2 + 5-Azacytidine (gray color), $p < 0.05$.

105 days in NO_2, NO_2 + 5-Azacytidine, and normal air group. IL-6 had no statistically significant difference between any of them in both BALF and plasma ($p > 0.05$ for all). Similarly, TNF-α level also had a similar tendency in both BALF and plasma ($p > 0.05$ for all) in Fig. 7.

4. Discussion

This study evaluated the characteristics of an emphysema model induced by chronic NO_2 exposure and explored the effects of demethylation on lung function and blood tests. The findings suggest that serial-days of exposure to 20 ppm NO_2 had the potential to cause non-inflammatory emphysema with global DNA hypomethylation in rat. Demethylation therapy with 5-Azacytidine improved both pulmonary function and Δweight, ameliorated the symptoms of hypoxia with reduced RBC, HGB, and HCT concentrations (chronic NO_2 exposure led to elevated levels of RBC, HGB, and HCT compared to normal air exposure; see Fig. S3).

Until now, various animal models have been created in an attempt to reproduce human COPD; however, but there are still some controversial and divergent aspects in these models. To the best of our knowledge and based on an extensive literature review, none of the currently available models can fully mimic the features of human COPD. Thus, it is necessary to establish useful and effective models that can mimic specific characteristics of different subtypes of human COPD. At present, three studies have identified the development of emphysema models in mice/rats without significant collections of inflammatory cells [15–17]. One of these models used a single intratracheal injection of active caspase-3 to induce emphysematous changes [15]. This model yielded direct evidence that alveolar wall apoptosis could result in pulmonary emphysema, but with a remarkable lack of inflammation [18]. The second model, induced via intravascular administration of a vascular endothelial cell growth factor receptor-2 (VEGFR-2) blocker, also caused non-inflammatory emphysema [16]. VEGF is a necessary component for endothelial cell survival and chronic VEGFR-2 blockade has been reported to generate alveolar septal cell apoptosis and airspace enlargement [19]. In addition, Petrache et al. demonstrated ceramide as a pivotal mediator of alveolar inflammation in emphysema [17]. Intratracheal instillation of ceramide in mice induced emphysema, while inhibition of enzymes that control de novo ceramide synthesis prevented alveolar cell apoptosis [15,17]. In the

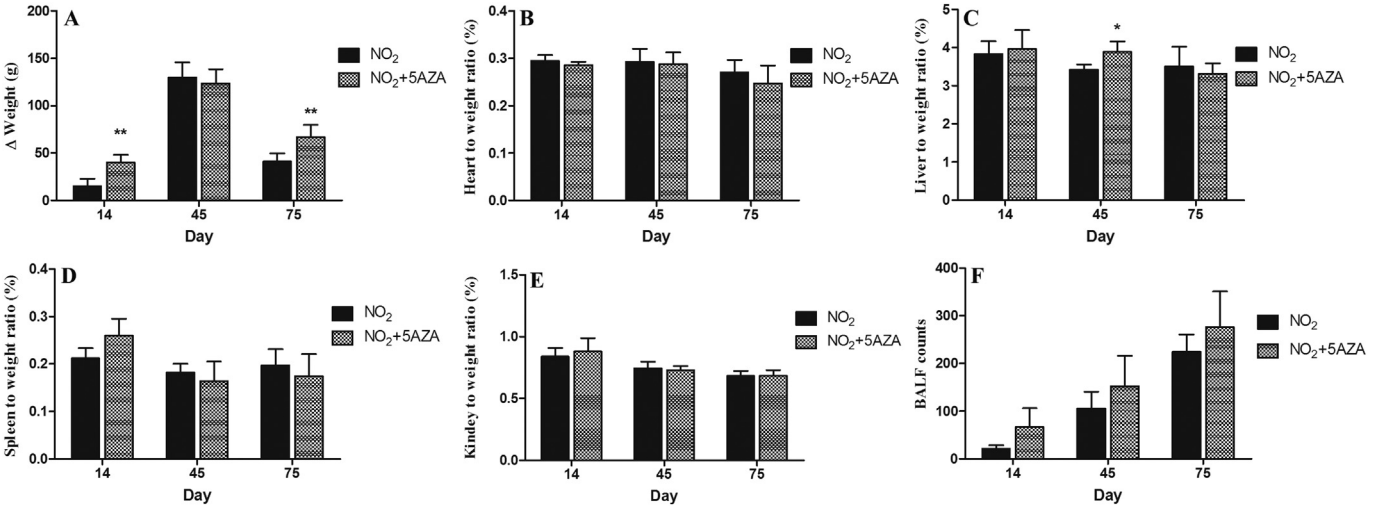

Fig. 4. Histograms (A–E) show Δweight and ratio of different organs (heart, liver, spleen, and kidney, respectively) to their own weight at different time points (14, 45, and 75 days) in NO_2 group compared to the NO_2 + 5-Azacytidine group. Histogram F shows inflammatory cell counts in BALF at different time points (14, 45, and 75 days) in the NO_2 group compared to the NO_2 + 5-Azacytidine group by using two-way ANOVA with Bonferroni correction. For NO_2 exposure (black color) vs NO_2 + 5-Azacytidine (gray color), $p < 0.05$.

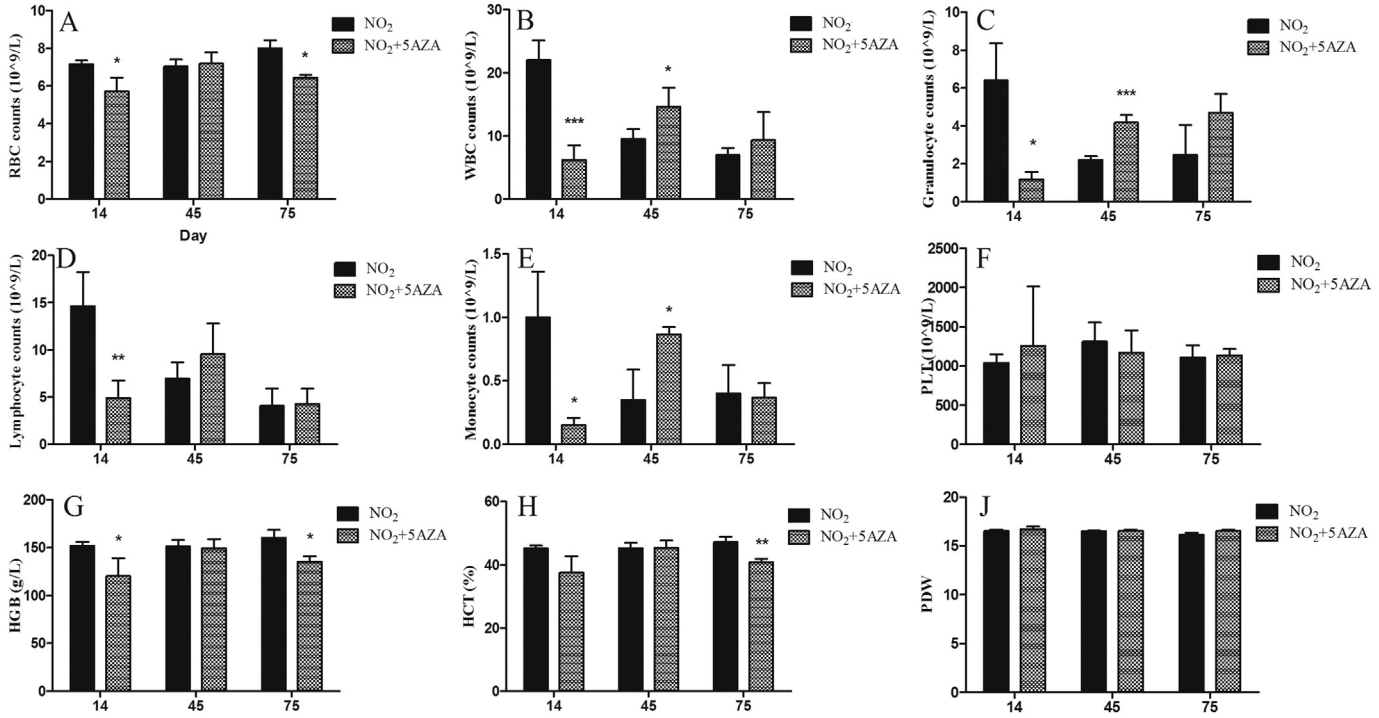

Fig. 5. Hematological index (RBC, WBC, Granulocyte, Lymphocyte, Monocyte, PLT, HGB, HCT, and PDW) in rats at different time points (14, 45, and 75 days) in the NO_2 group compared to the NO_2 + 5-Azacytidine group. For NO_2 exposure (black color) vs NO_2 + 5-Azacytidine (gray color), $p < 0.05$.

present study, chronic exposure of 20 ppm NO_2 for 75 days also led to emphysema in SD rats, but without the obvious accumulation of inflammatory cells. IL-6 and TNF-α levels in BALF and plasma, CD68 macrophages immunofluorescent staining, and inflammatory cell counts in BALF showed no statistically significant differences between the NO_2 exposure group and the normal air control group at each investigated time point. These data confirmed that emphysema induced by NO_2 chronic exposure could be a robust animal model for the study of the factors that lead to non-inflammatory emphysema in susceptible individuals.

Global DNA hypomethylation causes genomic instability, e.g., through chromatin structure modelling [20] and increased oncogene activation [21]. Epigenetic evidence indicates an inverse association between global methylation and exposure to air pollutants [22–24]. A meta-analysis in particular identified statistically significant average hypomethylation associated with NO_2 in human genome-wide DNA methylation [25]. This study focused on rats that were exposed to NO_2, and global DNA hypomethylation was also identified. Furthermore, given the remarkable role of epigenetics in COPD, DNA hypermethylation in specific genes might provide answers to the questions that could not be entirely answered by DNA sequence in COPD. Examples are the variable disease susceptibility in smokers and persistent adverse effects after smoking cessation [26]. 5-Azacytidine is a nucleoside-based DNA methyltransferase (DNMT) inhibitor that induces both demethylation and gene reactivation. It is an analog of cytosine, which is metabolically activated in vivo and readily incorporated into DNA during replication [8,27,28]. This action depletes soluble DNMT protein levels, leading to both replication-dependent global demethylation and gene activation [29,30]. 5-Azacytidine could alleviate the lung cell apoptosis and function failure generated by CSE. In vitro studies have

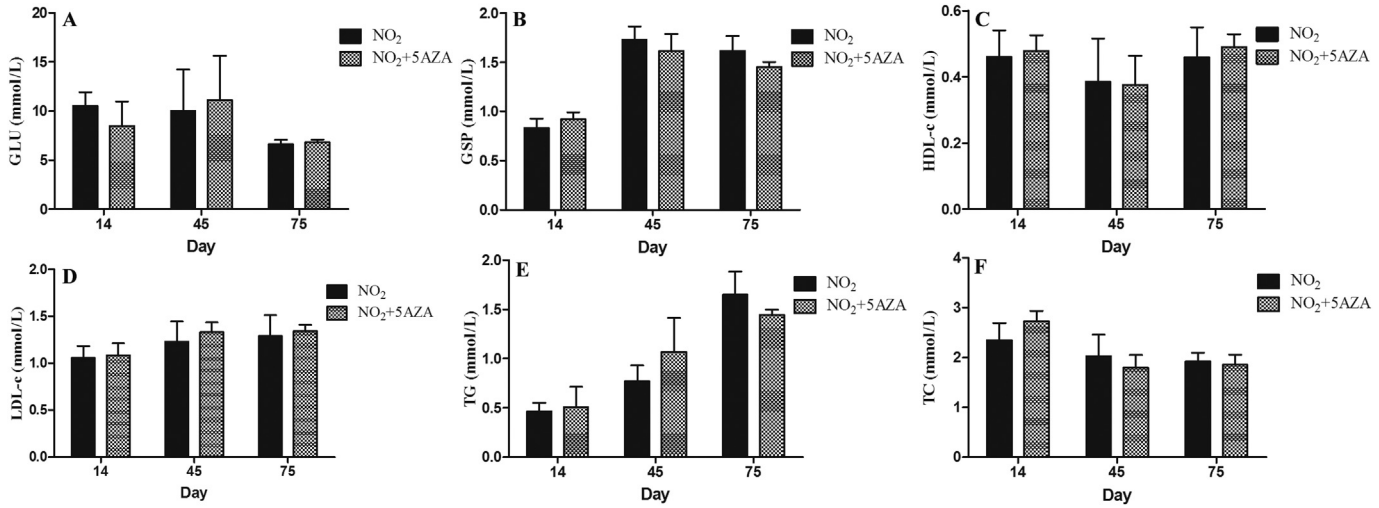

Fig. 6. Histograms (A-F) show biochemical indexes (GLU, GSP, HDL-C, LDL-C, TG, and TC, respectively) in the NO_2 group compared to the NO_2 + 5-Azacytidine group at different time points (14, 45, and 75 days). For NO_2 exposure (black color) vs NO_2 + 5-Azacytidine (gray color), $p < 0.05$.

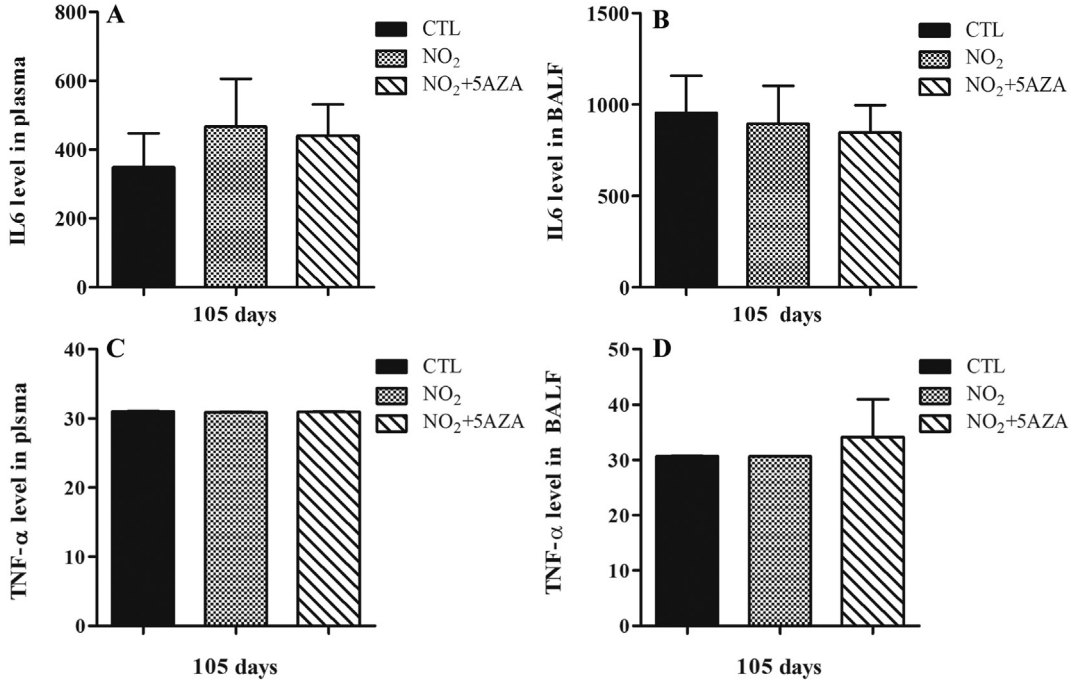

Fig. 7. Histograms (A–D) showing cytokine levels (IL-6 and TNF-α) of BALF and plasma in rats exposed to NO_2, NO_2 + 5-Azacytidine and normal air at 105 days. For NO_2 exposure (gray color) or NO_2 + 5-Azacytidine (stipes) vs normal air (black color), $p > 0.05$; or NO_2 exposure (gray color) vs NO_2 + 5-Azacytidine (stipes), $p > 0.05$.

shown that DNA demethylation with 5-Azacytidine increases the efferocytosis capacity and rescued the cells from CSE-induced defects in efferocytosis in a dose-dependent manner. According to these results, it seems plausible that demethylation protects smokers from emphysema, suggesting it as a promising treatment strategy for COPD. The present study demonstrated the protective effects of 5-Azacytidine on pulmonary function caused by NO_2 exposure. 5-Azacytidine also could result in restored Δweight at 14 and 75 days of NO_2 inhalation, and significantly reverse the effect of NO_2 exposure on Δweight compared to the normal air control group. NO_2 is an important environmental risk factor for declined pulmonary function and potentially impacts the methylation patterns of individual genes [31]. Therefore, treatment with 5-Azacytidine unexpectedly alleviated the decline of pulmonary function. The same tendency of changes in RBC, HGB, and HCT concentrations was also found (i.e., reduced levels in the NO_2 + 5-Azacytidine group). NO_2-mediated DNA hyper-methylation might participate in pathophysiological processes of emphysema, and conditions associated with emphysema could be ameliorated by demethylation. Underlying mechanisms should be further explored, and particularly the specific genes with the greatest influence on emphysema should be assessed.

Epigene-environment (such as air pollution or NO_2) interactions on lung function might be the one of important reasons to explain the effects on emphysema. The underlying mechanisms linking air pollution and lung function are not fully characterized. Lung function has been shown to be strongly heritable, part of which is controlled by inflammatory genes, oxidative stress and innate immunity mediators, such as CRP [32], IL-6 [33], INOS [34], or TLRs [35]. Other studies have reported stronger associations between air pollution and pulmonary outcomes in subjects with such polymorphisms [36,37]. There is increasing evidence that epigenetic mechanisms may interact with genetic variation to influence disease pathogenesis and the inheritance of disease traits. Johanna Lepeule et al. [38] have observed stronger associations of air pollutants with FVC than with FEV1, suggesting that air pollution may affect more of the smaller airways and therefore is associated with restrictive lung diseases [38].

NO_2 has relative insolubility (0.037 mL mL H_2O^{-1} at 35 °C), thus a large fraction of the inhaled NO_2 can be deposited in pulmonary alveoli [39]. Based on documented data, 70% of the NO_2 absorbed in the lungs is converted to nitrite acids (NO_2^-) but not nitrate acids (NO_3^-). Furthermore, it has been reported that the toxicity of NO_2 is mainly caused by the reaction of these oxidizing acids with readily oxidizable pulmonary and extra-pulmonary tissues instead of water [40]. The inhaled NO_2 could bind to lung tissue components and subsequently spread to the circulating medium. Consequently, it can damage the lungs in the following ways: 1) NO_2 can be converted to NO_2^- and NO_3^- in distal airways, and it has the ability to directly the damage in certain structural and functional lung cells; 2) NO_2 initiates the generation of free radical, which mainly causes protein oxidation, lipid peroxidation, and cell membrane damage; and 3) it reduces the resistance to infection by changing macrophage and immune function [41]. Therefore, in addition to its influences on pulmonary function, systemic effects are likely with NO_2, e.g., effects on the blood.

5. Conclusions

In summary, the findings of this study suggest that NO_2 exposure causes non- inflammatory emphysema with global DNA hypomethylation. The demethylation effect of 5-Azacytidine has the potential for improvements of pulmonary function, Δweight, and the symptoms of hypoxia. Methylation expression in specific genes as a result of NO_2 exposure should be deeply studied in future.

Abbreviations

NO_2	nitrogen dioxide
SD	Sprague-Dawley
WBC	white blood cell
RBC	red blood cell
HGB	hemoglobin
HCT	hematocrit
HDL-C	high-density lipoprotein cholesterol
LDL-C	low-density lipoprotein cholesterol
GLU	glucose

TC	total cholesterol
PLT	platelets
TG	triglyceride
GSP	glycated serum protein
FRC	functional residual capacity
TLC	total lung capacity
FVC	forced vital capacity
FEV1	forced expiratory volume in 1 s
Cchord	chord compliance

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Declaration of interest

None.

Author contributions

Study concept and design: JW and WJL; data acquisition: FL and ZLZ; analysis and interpretation of data: ZLZ; drafting of the manuscript: ZLZ and WJL; critical revision of the manuscript for important intellectual content: WJ, FL, and KY; statistical analysis: ZLZ and FL; administrative, technical, or material support: LY, MJD, LDC, JLY, and JQ; study supervision: LY and MJD.

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