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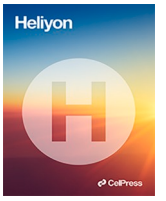

Nicotine plays a protective role in rats with induced viral pneumonia with polyinosinic-polycytidylic acid through α7nAChR

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ABSTRACT

Objective: To study the effect of nicotine in rat model of pneumonia induced by polyinosinic-polycytidylic acid [Poly (I:C)] and explore the underlying mechanism.

Methods: Twenty-four healthy adult male Sprague-Dawley (SD) rats (200–250 g) were randomly divided into normal saline control group (NS group); Poly (I:C) group; nicotine group (NIC group); and α7 nicotinic acetylcholine receptor (α7nAChR) antagonist group (α-BGT group) (n = 6 each). Rats in the Poly (I: C), NIC, and α-BGT groups were administered 1.5 mg/mL 100 μL Poly (I:C) intranasally to establish pneumonia model. In α-BGT group, 1 μg/kg α-bungarotoxin (α-BGT) was intraperitoneally injected 45 min before intranasal Poly (I:C), and 400 μg/kg nicotine was intraperitoneally injected 15 min after α-BGT injection. The NIC group received an equal volume of NS in place of α-BGT while the other treatments were same. The Poly (I:C) group received equal volume of NS in place of nicotine while the other treatments were same as in NIC group. In the NS group, only NS was administered at all three time points. $PaCO_2$, PaO_2, and PaO_2/FiO_2 levels were determined 24 h after administration of Poly (I:C). After euthanization, rat lung tissues were extracted for pathological examination, and wet weight/dry weight (W/D ratio) was determined. Expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1β, and interferon (IFN)-γ in lung tissue was determined by ELISA. q-PCR was used to detect nuclear factor kappa-B P65 (NF-κBP65).

Results: Compared with NS group, Poly (I:C) and α-BGT groups showed significantly increased W/D ratio, $PaCO_2$, TNF-α, IL-6, IL-1β, and IFN-γ content, NF-κB P65 expression, and reduced PaO_2 and PaO_2/FiO_2 ($p < 0.05$), along with obvious signs of pathological injury. Nicotine pre-treatment reduced W/D ratio, $PaCO_2$, proinflammatory cytokines, NF-κBP65 expression, and increased PaO_2 and PaO_2/FiO_2 levels. The above effects were negated in α-BGT group.

Conclusion: Pre-administration of nicotine improved Poly (I:C)-induced pneumonia by activating the cholinergic anti-inflammatory pathway.

1. Introduction

Viral pneumonia is a common and potentially fatal infectious disease. Over the last two decades, there have been three major

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outbreaks of viral pneumonia associated with high mortality, i.e., coronavirus disease 2019 (COVID-19), Middle East respiratory syndrome, and severe acute respiratory syndrome (SARS). The clinical manifestations and severity of viral pneumonia show considerable inter-individual differences based on the underlying condition of the patient. Patients with tumors or other autoimmune diseases and elderly patients have high morbidity and mortality [1].

When virus infects the body and invades the lung tissue, it causes the patient's immune system to react violently and produce a "cytokine storm". Failure of the immune system to resist virus invasion may induce acute respiratory distress syndrome (ARDS). Further aggravation may cause systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and death [2].

Innate immune cells of patients with severe COVID-19 often show upregulation of genes that promote inflammatory responses downstream of NF-κB and type I interferon signaling pathways [3]. Peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers showed higher expression of proinflammatory cytokines after infection with SARS-CoV-2 (including tumor necrosis factor [TNF], interleukins (IL-6, IL-1β), and interferon γ [IFN-γ]) [4]. In previous studies on patients with COVID-19, cytokine storm was significantly associated with lung injury, MODS, and mortality. High expression of TNF and IFN-β in patients with severe COVID-19 was especially associated with significant increase in the incidence of ARDS, MODS, and mortality [5,6].

Despite the advances in the development of anti-inflammatory drugs, further research is required to reduce lung damage and patient mortality [7]. Nicotine is an agonist of alpha-7 nicotinic cholinergic receptor (α7nAChR). Previous studies have suggested anti-inflammatory effects of nicotine which are mainly mediated via activation of cholinergic anti-inflammatory pathways. Nicotine can act on α7nAChRs expressed by alveolar macrophages and lung epithelial cells, thereby alleviating inflammatory changes in lung tissue [8,9]. In addition, some studies have found lower prevalence rates of smoking among COVID-19 patients requiring hospitalization, which suggests a potential protective effect of nicotine in the setting of viral pneumonia [10]. However, there is no conclusive evidence of the effect of nicotine on viral pneumonia.

The aim of this study was to explore the effect of nicotine in a rat model of pneumonia. Our findings suggest that nicotine inhibited the activation of NF-κB signaling pathway through α7nAChR, leading to reduced production of pro-inflammatory cytokines. This suggests a potential therapeutic role of nicotine in reducing the inflammatory response in patients with COVID-19.

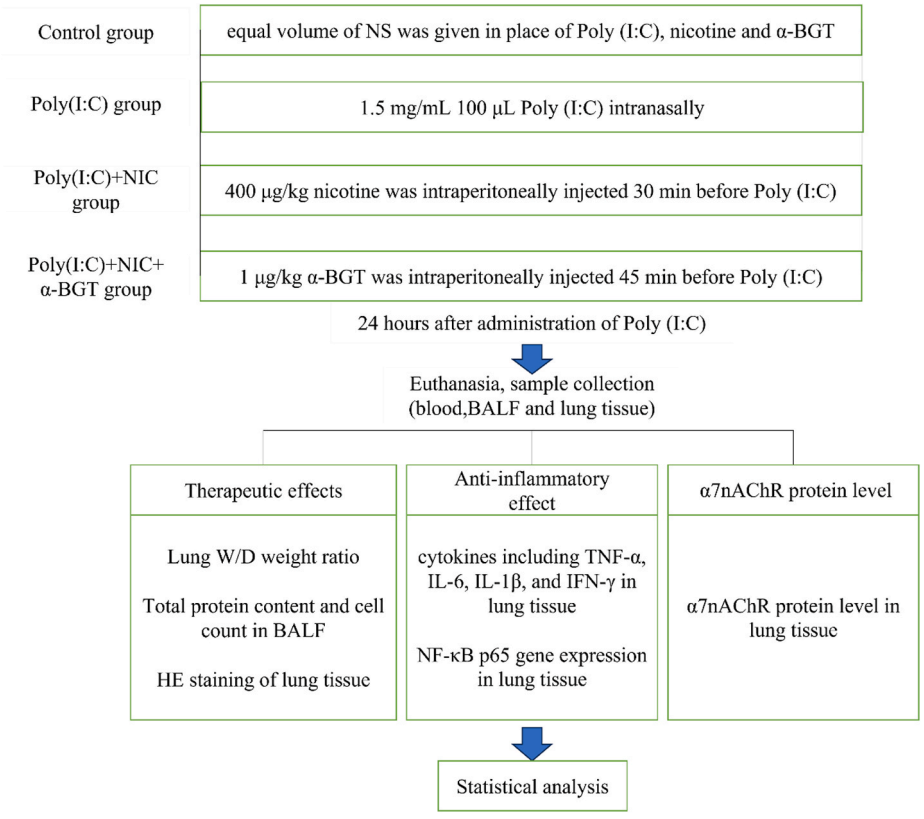

Fig. 1. Overview of the experimental design for all groups.

2. Materials and methods

2.1. Reagents

Nicotine was obtained from Shanghai Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). Poly (I: C) was obtained from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). α-bungarotoxin (α-BGT) was obtained from Shanghai Hengfei Biotechnology Co. Ltd. (Shanghai, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-1β, TNF-α, and interferon (IFN)-γ were purchased from Wuhan ELK Biotechnology Co. Ltd. (Wuhan, China) to examine IL-6, IL-1β, TNF-α, and IFN-γ levels in lung tissue.

2.2. Rat viral pneumonia models

Twenty-four healthy male Sprague-Dawley (SD) rats (200–250 g) purchased from the Animal Experiment Center of Wuhan University (Wuhan, China) were randomly divided into the following four groups: normal saline control group (NS group); Poly (I: C) group; nicotine group (NIC group); and α7nAChR antagonist group (α-BGT group). In the NS group, rats were anesthetized with pentobarbital sodium (50 mg/kg) and administered intranasal normal saline. In the Poly (I: C) group, rat model of viral pneumonia was established by intranasal administration of 1.5 mg/mL 100 μL poly (I: C). In NIC group, 400 μg/kg nicotine was injected intraperitoneally 30 min before the establishment of viral pneumonia model. Rats in the α-BGT group received intraperitoneal injection of α-BGT 45 min before the establishment of viral pneumonia model. Twenty-four hours after administration of Poly (I: C), rats were anesthetized with pentobarbital sodium (50 mg/kg), blood samples from the abdominal aorta were collected. After the rats were sacrificed by bloodletting through the abdominal aorta, alveolar lavage fluid (BALF) and lung tissue samples were obtained (Fig. 1). All experimental operations complied with the principles in the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006). This study was approved by the Animal Ethics Committee of Wuhan University (approval number: WP20220054).

2.3. Measurement of cell counts and protein in BALF

BALF was obtained after injection of 2 mL of cold PBS into the left bronchus of rats through the oropharynx and main trachea with a syringe. The BALF specimens were centrifuged at 2000 rpm at 4 °C. The total protein concentration in the supernatant was determined by BCA protein assay. The cell types and counts in the sediment were calculated using a CASY TT Cell Counter and Analyzer System (OMNI Life Science, Germany).

2.4. Quantitative arterial blood gas analysis

Two mL of arterial blood was taken from the abdominal aorta of rats. Within 5–10 min, blood samples were placed in a blood gas analyzer (ABL700, Roche, Switzerland) to measure oxygen partial pressure (PaO_2), carbon dioxide partial pressure ($PaCO_2$), and oxygenation index (PaO_2/FiO_2 ratio).

2.5. Determination of acute lung injury score

Lung tissue extracted from rats were fixed, dehydrated, embedded, and sliced. Sectioned specimens were stained with hematoxylin and eosin (HE). Pathological evaluation was performed according to the American Thoracic Society Acute Lung Injury (ALI) Scale [11].

2.6. Quantification of wet/dry weight ratio

Lung tissue was isolated from rats, placed on weighing paper for weight measurement, and the result was recorded as wet weight (W). Then, the lung tissue was heated in a constant temperature oven at 80 °C for 48 h, and placed on a new weighing paper for weight measurement, and the result was recorded as dry weight (D). Subsequently, the W/D ratio was calculated.

2.7. Cytokine analysis by ELISA

Rat lung tissue was grounded and the tissue homogenate was centrifuged at 400 g for 20 min at 4 °C. The supernatant was quantified on a microplate reader for cytokines including TNF-α, IL-6, IL-1β, and IFN-γ (ELK Biotechnology Co. Ltd., Wuhan, China).

Table 1
Primer sequences.

Target genes	Primer sequences (5′–3′)	
β-ACTIN [12]	For:	CGTTGACATCCGTAAAGACCTC
	Rev:	TAGGAGCCAGGGCAGTAATCT
NF-κB p65 [13]	For:	TCTGCCGAGTAAACCGGAAC
	Rev:	CAGGCTAGGGTCAGCGTATG

2.8. RNA extraction and q-PCR

RNA was extracted from lung tissue homogenate and M-MLV Reverse Transcriptase kit (ELK Biotechnology, China) was used according to manufacturer's guidelines for reverse transcription. Total RNA was extracted using Tripure reagent (ELK Biotechnology, China). β-Actin was used as an internal control. NF-κB p65 gene expression was determined using ΔΔ Ct quantitative method. Quantitative real-time PCR was conducted on the Step One Real-Time PCR System (Applied Biosystems, CA) using QuFast SYBR Green PCR Master Mix (ELK Biotechnology, China). The sequence of β-Actin and NF-κ B p65 primers are shown in Table 1.

2.9. Western blot analysis

Rat lung tissues were extracted, homogenized, lysed, and centrifuged to obtain total protein as reported previously [14]. After separation by SDS-PAGE, protein samples were transferred onto a PVDF membrane (Millipore, USA). Next, the PVDF membranes were incubated overnight with primary antibodies including α7nAChR (1:1000, #21379-1-AP, Proteintech, China) and β-actin (1:5000, #20536-1-AP, Proteintech, China). This was followed by incubation with the secondary antibody (1:5000; #BA1054, Boster, China) for 30 min. The intensity of protein bands was detected and quantified using the ChemiDocXRS + chemiluminescence detection system in the Image Lab software (Bio-Rad, USA).

2.10. Statistical analysis

The data were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance followed by Tukey's post hoc test. All statistical analyses were performed using GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA.) P values < 0.05 were considered indicative of statistical significance.

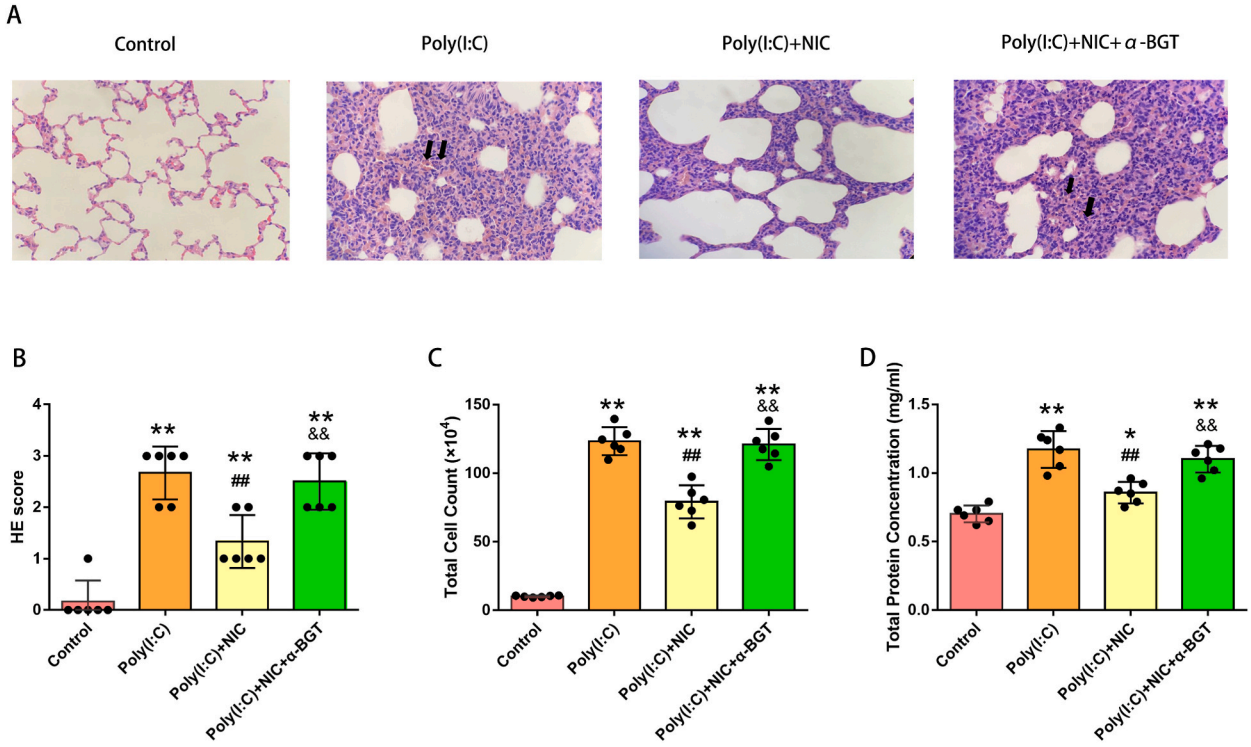

Fig. 2. Effects of nicotine on Poly (I: C)-induced changes in lung tissue. (A) HE-stained sections of lung tissues in the various groups (400 ×). Arrow indicates inflammatory cell infiltration and red blood cell exudation. (B) Quantitative analysis of HE scores; (C) Compared to the Poly (I:C) group (123.3 ± 10.17; n = 6), the total cell count in the NIC group (79.10 ± 12.03; n = 6) was significantly decreased. (D) Compared to the Poly (I:C) group (1.172 ± 0.134; n = 6), the total protein concentration in the Nic group (0.857 ± 0.078; n = 6) was significantly decreased. Data presented as mean ± standard deviation. *p < 0.05 versus control group; **p < 0.01 versus control group; ##p < 0.01 versus Poly (I:C) group; &&p < 0.01 versus Poly (I:C) + NIC group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3. Results

NIC inhibited pathological process of poly (I:C)-associated pneumonia in rats HE sections of lung tissues in the various groups and the quantitative analysis of HE scores are shown in Fig. 2A and B, respectively. Compared to NS group, Poly (I:C) group displayed typical features of lung injury, including alveolar wall widening, edema, alveolar collapse, extensive inflammatory cell infiltration, and red blood cell exudation. Histopathological damage as well as the HE score were significantly attenuated in the NIC group (Fig. 2B). Compared to the NS group, total cell count and total protein concentration in the Poly (I:C) group were significantly increased ($p <$ 0.01, respectively, Fig. 2C and D). In the NIC group, BALF cell count and protein levels were decreased. However, α-BGT treatment blocked the effects of NIC ($p < 0.01$, respectively, Fig. 2A–D).

3.1. NIC ameliorated inflammatory response in the lung tissues of rats with induced viral pneumonia

Compared with the NS group, the poly (I:C) group had significantly higher levels of pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, and IFN-γ ($p < 0.01$, respectively, Fig. 3A–D). However, these cytokines were markedly reduced after treatment with NIC. The cytokine levels in the NIC group were significantly lower than those in the Poly (I:C) group. Nevertheless, these effects were reversed by α-BGT ($p < 0.01$, respectively, Fig. 3A–D).

3.2. NIC alleviated poly (I:C)-induced acute lung injury in induced viral pneumonia rats

The results of blood gas analysis were normal in the NS group, while Poly (I:C) group showed significantly declined PaO_2 level and PaO_2/FiO_2 ($p < 0.01$, respectively, Fig. 4A, C), and elevated $PaCO_2$ level ($p < 0.01$, Fig. 4B). After administration of nicotine, $PaCO_2$ decreased while PaO_2 and PaO_2/FiO_2 increased in rats with induced viral pneumonia. Conversely, α-BGT treatment offset the anti-

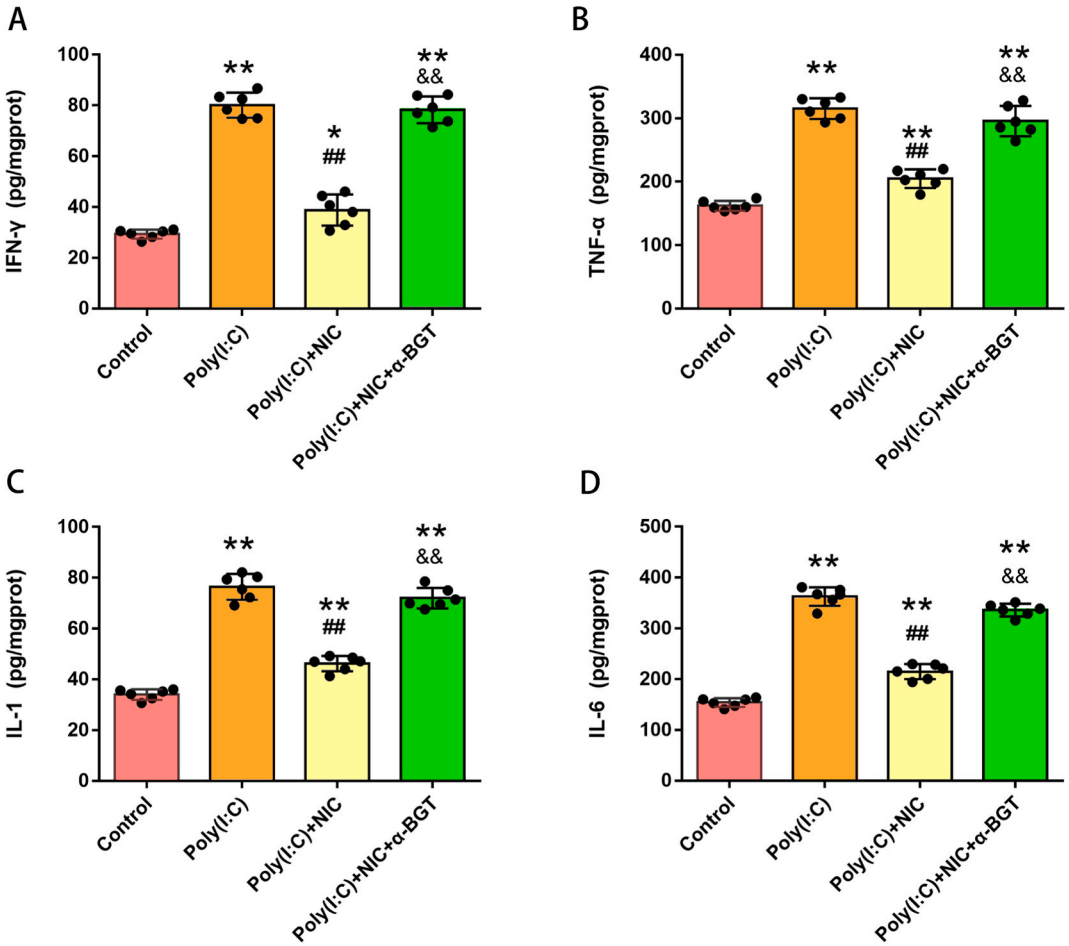

Fig. 3. Effects of nicotine on Poly (I: C)-induced cytokines in lung tissue. IFN-γ (A), TNF-α (B), IL-1β (C), IL-6 (D) (n = 6). Data presented as mean ± standard deviation. *p < 0.05 versus control group; **p < 0.01 versus control group; ##p < 0.01 versus Poly (I:C) group; &&p < 0.01 versus Poly (I: C) + NIC group.

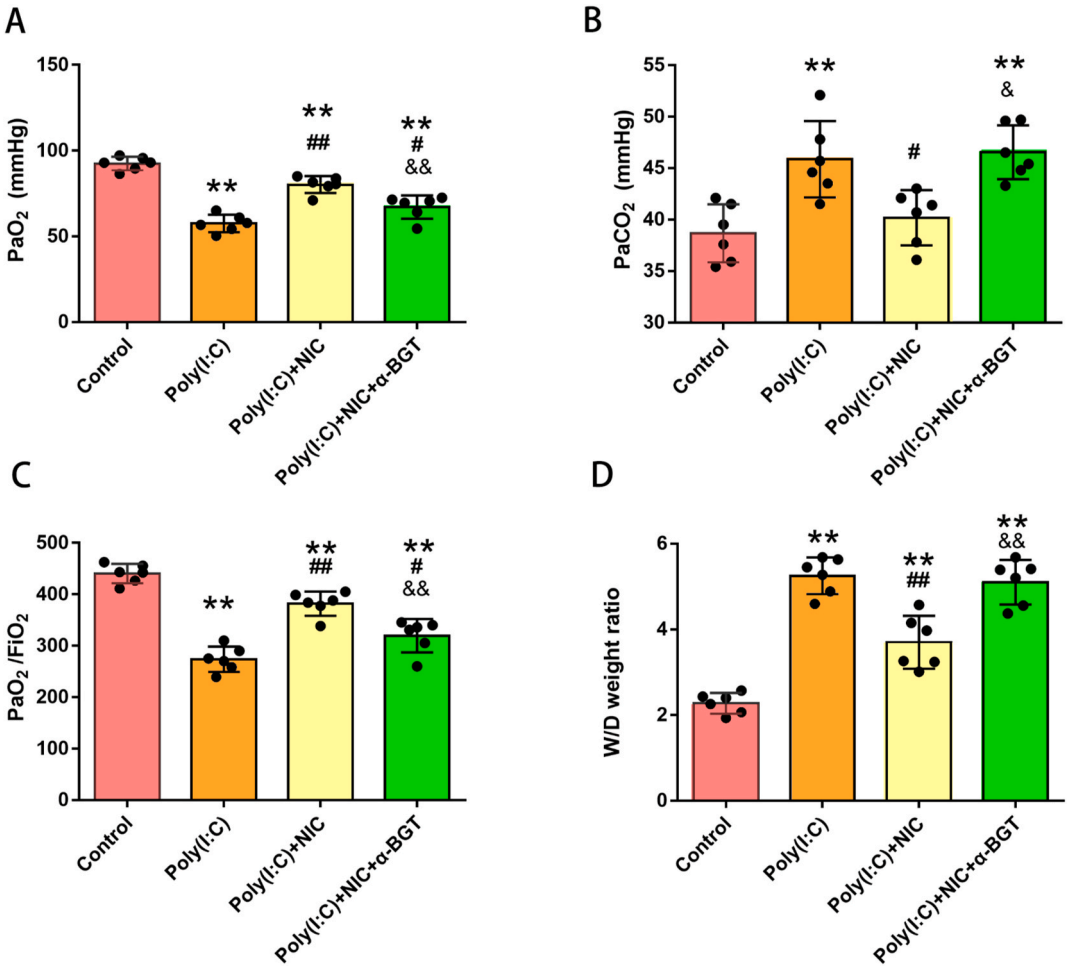

Fig. 4. Effects of nicotine on Poly (I: C)-induced changes in arterial blood gas indices and Wet/Dry weight ratio. (A–C) Results of arterial blood gas analysis in the various groups **(n=6)**; (B) Wet/Dry weight ratio in the various groups **(n=6)**. Data presented as mean ± standard deviation. **p < 0.01 versus control group; #p < 0.05 versus Poly (I:C) group; ##p < 0.01 versus Poly (I:C) group; &p < 0.05 versus Poly (I:C) + NIC group; &&p < 0.01 versus Poly (I:C) + NIC group.

inflammatory effect of nicotine. Rats with induced viral pneumonia presented pulmonary edema manifested by an increased W/D ratio. However, NIC treatment significantly improved the pulmonary edema caused by Poly (I:C), an effect that was also reversed by α7nAChR pretreatment ($p < 0.01$, Fig. 4D).

3.3. *Effect of NIC on NF-κB p65 and α7nAChR*

To further explore the mechanism of the protective effect of nicotine in induced viral pneumonia, we performed quantitative analysis of NF-κB p65 and α7nAChR expression in the lung tissue of rats with viral pneumonia. Rats with induced viral pneumonia showed significantly increased expression of NF-κB p65 and significantly decreased expression level of α7nAChR in the lung tissue ($p < 0.01$ for both Fig. 5A and B; The original uncropped figure is detailed in the Supplementary Fig. 1). Nicotine was found to alleviate the severity of viral pneumonia. However, pretreatment with α7nAChR negated this protective effect.

4. Discussion

In this study, we investigated the effects of nicotine on induced viral pneumonia in a rat model and explored the underlying mechanisms. The results suggest that nicotine may reduce the occurrence of Poly (I:C)-induced lung injury in rats by stimulating α7nAChR.

Treatment of viral pneumonia is a contemporary research hotspot owing to the large scale outbreaks of viral pneumonia across the world. Since Toll-like receptor (TLR-3) is involved in the pathogenesis of viral pneumonia, the specific ligand Poly (I:C) of TLR-3 is usually used to establish the viral pneumonia model [15]. TLR-3, a member of TLR family proteins, recognizes extracellular dsRNA and

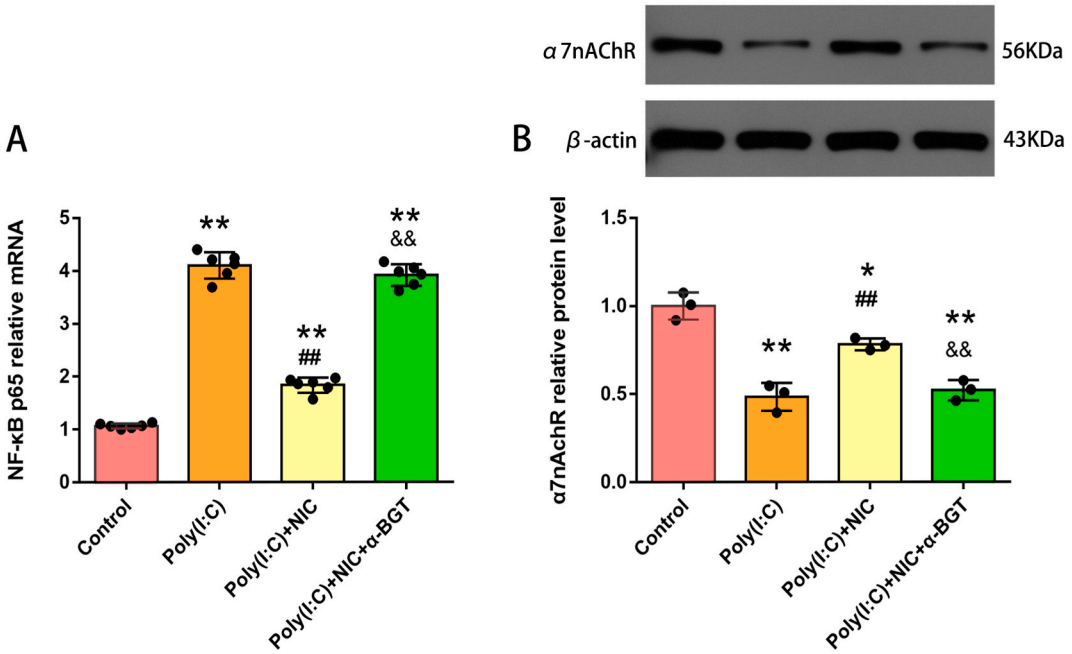

Fig. 5. Effects of nicotine on Poly (I: C)-induced NF-κB p65 gene expression in lung tissue (A) and α7nAChR protein level (B) in the various groups. NF-κB p65 gene expression in lung tissue (A) **(n=6)** and α7nAChR protein level (B) **(n=3)**. Data presented as mean ± standard deviation. *p < 0.01 versus control group; **p < 0.01 versus control group; ##p < 0.01 versus Poly (I:C) group; &&p < 0.01 versus Poly (I:C) + NIC group.

activates NF-κB [16]. TLR3 ligand poly (I:C)-induced mice model of pneumonia was characterized by overproduction of pro-inflammatory cytokines through NF-κB signaling pathway [17]. TNF-α, IL-1, and IL-6 have been implicated as key cytokines in cytokine storm. IFN- γ is also an important immune regulatory factor.

Previous studies have shown that the vagus nerve can regulate the release of TNF, thereby attenuating inflammation. This suggests that cholinergic pathways may inhibit the occurrence of inflammatory responses. Since the early 2000s, animal experiments have shown that stimulation of the cholinergic system and the parasympathetic vagal system reduces the expression of inflammatory cytokines [18]. As one of the nicotinic receptors expressed on macrophages, α7nAChR plays an important role in regulating cholinergic anti-inflammatory pathways and cytokine production [19]. Acetylcholine (or nicotine) has been shown to inhibit the activation of NF-κB pathway and the production of proinflammatory cytokines, including TNF, IL-1β, IL-6, and IL-18, by binding to α7nAChR [18,19]. Kanauchi et al. demonstrated that nicotine and vagus nerve stimulation may inhibit the occurrence of oxazolone-induced Th2 colitis by activating α7nAChRs expressed on plasmacytoid dendritic cells (pDCs) [20]. Fujii et al. showed that nicotine can modulate immune responses by altering cytokine production, while the α7nAChR signaling pathway modulates immune responses by modifying T cell differentiation [21]. These findings suggest that the anti-inflammatory effect of nicotine may be attributable to the activation of the cholinergic anti-inflammatory pathway via stimulation of a7nAChRs. Nicotine therapy was shown to reduce endotoxin-induced leukocyte infiltration and edema in lung tissue, and down-regulate the production of pro-inflammatory chemokines and cytokines in lung tissue [8]. Then we found that nicotine may improve the pathophysiology of the viral pneumonia, decreasing NF-κBP65 formation and regulating cytokine production.

In this same study, nicotine was found to down-regulate inflammatory changes in lung tissue by acting on α7nAChR expressed by alveolar macrophages and lung epithelial cells [8]. A clinical study suggested a potential protective effect of nicotine against COVID-19 based on the smaller proportion of smokers among patients with symptomatic COVID-19 [22]. Cui et al. found that nicotine can significantly attenuate the poly (I:C)-induced molecular response of macrophages [23]. However, whether nicotine has a protective effect against the occurrence of pneumonia caused by inflammatory factor storm induced by viral infection is not clear. In this study, nicotine 400 μg/kg was injected intraperitoneally 30 min prior to the administration of Poly (I:C). The lung tissue changes in the nicotine group were reduced, and only part of the pulmonary interstitium showed mild inflammatory cell infiltration, indicating less lung damage compared to the Poly (I:C) group. The nicotine pretreatment group showed decrease in the W/D ratio, expression of NF-κBP65 and inflammatory factors in lung tissue, the bronchoalveolar lavage fluid the total cell count, protein levels, and $PaCO_2$, and increase in PaO_2 and PaO_2/FiO_2.

α7nAChR has been shown to be an important component of the anti-inflammatory cholinergic signaling pathway. Studies have shown that the cytokine storm caused by COVID-19 reduced the expression of α7nAChR which negatively regulates the inflammatory response [24]. In addition, nicotine was shown to increase the expression of α7nAChR [25]. After establishment of the rat pneumonia model, the formation of α7nAChR in lung tissue was significantly reduced. However, this effect was reversed after administration of nicotine. In order to explore the protective mechanism of nicotine against pneumonia, we used the α7nAChR antagonist

α-bungarotoxin. In the α-BGT group, the α7nAChR expression in the rat lung tissue was decreased; however, there were many inflammatory cells; increased W/D ratio of lung; increased expression of NF-κBP65, and high levels of inflammatory factors in lung tissue. The total cell counts, protein levels were increased; $PaCO_2$ increased, PaO_2 and PaO_2/FiO_2 decreased. These findings suggested that α-BGT counteracted the ameliorating influence of nicotine on pulmonary changes, suggesting that the ameliorating effect of nicotine on the pulmonary changes was related to α7nAChR. Thus, Poly (I:C)-induced pneumonia can be prevented following activation of cholinergic pathways.

Cytokine storm may be the main cause of SIRS and MODS in patients with COVID-19. Some studies have shown that activating α7nAChR by physical or chemical means can regulate cholinergic pathways, which can reduce the excessive release of cytokines in patients with COVID-19. This could be a potential target for therapy in the future.

Activation or inhibition of cholinergic pathways may serve as potential regulatory targets that may affect cytokine release in patients with viral pneumonia. As nicotinic receptors, α7-nAChRs may reduce the severity of COVID-19 patients by regulating the release of pro-inflammatory factors and inhibiting the generation of cytokine storm [24,25].

In comparison to the prevalence of smoking in the general population, the proportion of hospitalised COVID-19 patients who are current smokers is significantly lower [4,5]. In a study, after controlling for covariates, smoking was found to be significantly associated with a reduced risk of hospitalization for COVID-19 [6]. While smoking itself cannot be supposed to have a protective effect against COVID-19, the potential of pharmaceutical nicotine products in this regard warrants further exploration, given their widespread availability. Nicotine has been proposed as a potential therapy to mitigate the inflammatory responses associated with viral pneumonia.

Some major limitations of this study should be noted. First, it is unknown whether agonist/antagonist of nicotine, such as varenicline or cytisine, plays a similar protective role against poly (I:C)-induced pneumonia by activating the cholinergic anti-inflammatory pathway. This should be further investigated owing to their lesser side effects. Second, although this study demonstrated the protective effect of NIC against poly (I:C)-induced pneumonia and identified the potential involvement of cholinergic anti-inflammatory pathway in mediating this protective effect, a further study involving a larger number of animals would provide more robust evidence. Thirdly, different doses of NIC should be further tested to justify the optimal dosage for its protective effect.

In conclusion, this study found that nicotine can alleviate the pulmonary changes by increasing the expression of α7nAChR and inhibiting the expression of NF-κBP65, thereby reducing the production of cytokines. It also suggested that activation of the cholinergic pathway has an ameliorating effect on the pulmonary changes induced by Poly (I:C). In combination with previous studies, nicotine can activate cholinergic anti-inflammatory reflex, thus decreasing of expression of pro-inflammatory cytokines, and improving lung disease. Therefore, we speculate that nicotine can reduce the inflammatory reaction in patients with viral pneumonia by activating the cholinergic pathway, which may become a potential treatment for COVID-19 and other viral infections.

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Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

CRediT authorship contribution statement

Wei Liu: Writing – review & editing, Writing – original draft, Data curation. **Yi Zhu:** Writing – review & editing, Software, Methodology, Formal analysis. **Hong Yan:** Writing – review & editing, Conceptualization. **Lingyun Ren:** Writing – review & editing, Data curation. **Jingli Chen:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e21667.

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