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Establishment of a mouse pneumonia model under cold stress

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Abstract

Simulating the cold living environment of the human body in winter, establishing a mouse model of pneumonia under single-factor cold stimulation to provide a basis and a new direction for further research on the relationship between cold and pulmonary inflammation. C57/BL6J mice were divided into three groups: A (initial control group), B (control group), and C (experimental group). They were randomly divided into 10 mice for each group, placed in an environment (temperature 22 ± 1 °C, humidity 50 ± 5%). After 3 weeks, mice in group A were sacrificed. Group C mice were exposed to environment (temperature 4 °C, humidity 50 ± 5%) for 8 h (9 am to 5 pm), and mice in group B were placed. (The temperature was 22 ± 1°C, humidity 50 ± 5%). The lungs of the mice in group A and B were normal. The inflammatory cells infiltrated in the mice in group C, and the alveolar structure disappeared. The neutrophil immunohistochemistry showed a significant increase in neutrophils and the percentage of blood neutrophils. Higher than group A and B ($P < 0.05$). The mice were stimulated daily for 8 hours at a temperature of 4 °C and humidity (50 ± 5) % for 30 days to establish a cold-stimulated pneumonia model.

Keywords: cold stimulation; pulmonary infection; model; mouse.

Practical Application: Establishment of a mouse pneumonia model under cold stress.

1 Introduction

Many epidemiological studies have provided evidence of the relationship between morbidity, mortality and environmental temperature (Åström et al., 2019; Yakovleva et al., 2019), and mortality caused by low temperature is much higher than that caused by high temperature (Gasparrini et al., 2015; Shor & Roelfs, 2019; Zhan et al., 2017). It has been suggested that the risk of respiratory diseases increases significantly when the external temperature decreases, especially in the elderly (Bunker et al., 2016). In recent years, some reports have shown that cold stress affects immune response, including changes in lymphocyte subsets (Ferreira et al., 2015; Kim et al., 2014). At the same time, it is also reported that cold stress directly affects lung tissue or cells (Franchini et al., 2016) through an independent way of system regulation, thus making them change. Although the relationship between environmental hypothermia and susceptibility to respiratory infection is generally accepted, the model of hypothermia as a single factor on lung inflammation to host has never been successfully established.

Pneumonia, as one of the most common diseases in clinic, is also one of the most common diseases leading to sepsis and multiple organ failure. The infection of Gram-negative bacteria is the main cause of acute lung injury, and lipopolysaccharide is the cell surface endotoxin of Gram-negative bacteria, which is the main activator of the inflammatory response leading to acute lung injury (Huang et al., 2021; Li et al., 2021). It is generally believed that cold stimulation is related to the susceptibility of respiratory system infection, and cold stimulation is only a secondary inducer (Joo et al., 2016). However, especially in the seasons with large temperature difference, the probabilities of respiratory diseases are directly and significantly increased, especially for children and the elderly with weak immunity in the cold environment of real life (Shakoor et al., 2021). Therefore, we believe that cold stimulation may be the main factor leading to host pneumonia directly. Through the cold environment stimulation, we take the mice as the experimental object, and then confirm the successful establishment of pneumonia model under the cold environment through the lung pathology, immunohistochemistry of neutrophils and the percentage of neutrophils in the blood so as to provide a basis for further research on the relationship between cold and pulmonary inflammation, then providing a new direction for the subsequent clinical effective prevention of pulmonary inflammation.

2 Materials and methods

2.1 Animal

C57BL/6J mouse (inbred line), male, clean grade, weight: 18-22 g (Shanghai SLAC Laboratory Animal Co.,Ltd; License No.: scxk (Shanghai) 2017-0005; Certificate No.: 20170005003934).

2.2 Drugs and reagents

First antibody: Ly6g antibody, RB6-8C5 (ab25377), Abcam company, English; DAPI (g1012) Servicebio Company, Wu Han, China; second antibody (FITC Goat anti rat) (gb22302) Servicebio Company, Wuhan, China; 5% chloral hydrate; toluidine blue

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dye solution (Solarbio, Beijing, China); EDTA antigen repair solution (Qcbio Science & Technologies Co., Ltd., Shanghai, China); hematoxylin eosin dye solution (Solarbio, Beijing, China).

2.3 Instruments

Sysmex XE-5000 automatic blood analyzer (SYSMEX, Japan); fluorescence microscope (Nikon, Japan); imaging system (Nikon DS-U3, Japan).

2.4 Experimental conditions

Thirty C57BL/6J male mice of 5-week-old were randomly selected and fed in the environment of (22 ± 1) °C and (50 ± 5)% humidity for 2 weeks. After that, 30 mice were randomly divided into three groups: initial control group (A group), control group (B group) and experimental group (C group), 10 mice for each group. For the initial control group, 10 mice were anesthetized with 5% chloral hydrate (0.07 ml/10 g) intraperitoneally, then the blood of orbital venous plexus was collected for routine blood test (Yang et al., 2015). After mice cervical spondylolisthesis and execution, the lungs were taken out under sterile operation and fixed in 4% paraformaldehyde. The control group was kept standard feeding in the environment of temperature (22 ± 1) °C, humidity of 50 ± 5%. The experimental group was exposed to the environment of temperature 4 °C, humidity (50 ± 5)% for 8 hours (9 a.m.-5 p.m.) every day, and then transferred to the environment of temperature 22 ± 1 °C, humidity of (50 ± 5)% for feeding. The respiratory rate, appetite and activity of mice in each group were observed once a day during the feeding process. After 30 days of repetition, most of the mice in group C had poor appetite and decreased activity, and some of them had shivering and dorsiflexion. Mice in Group B and Group C were anesthetized at the same time, blood and lung tissue samples were collected, then, lung tissues were fixation in 4% paraformaldehyde, and blood samples were immediately detected. All experimental protocols were approved by the Review Committee of Provincial Hospital Affiliated to Anhui Medical University.

2.5 Blood and lung specimens

The percentage of neutrophils in the blood of orbital venous plexus of mice was detected and the data was counted. All the data were analyzed by ANOVA. The lung tissues was embedded in paraffin and sectioned. Some paraffin sections were stained for lung pathology detection. A part of paraffin sections were dewaxed, and then the tissue sections were placed in a box filled with citric acid antigen repair buffer (pH6.0) for antigen repair in the microwave oven. The slides were placed in PBS (pH7.4) for 3 times, and the endogenous peroxidase was blocked for 5 minutes each time. Serum blocking and one antibody (ly6g antibody) was used, the sections were incubated overnight in a wet box at 4 °C. (added some water in the box to prevent evaporating). After adding the second antibody, adding DAPI to stain the nucleus.

2.6 Statistical analysis

The data were expressed by mean±standard deviation, and the comparison between groups was analyzed by one-way ANOVA ($P < 0.05$ means statistical difference).

3 Results

3.1 Percentage of neutrophils of three groups of mice

We set the initial control group as Group A, the control group as Group B, and the experimental group as Group C. The results of blood samples are shown in Table 1. P2 showed that there was no significant statistical difference between Group A and Group B, indicating that there was no correlation between age and pneumonia. There was significant statistical difference between Group B and Group C ($P < 0.001$), and there was also significant statistical difference between Group A and Group C ($P3 < 0.001$).

3.2 Pathological changes of lung tissues

The pathological results were shown in Figure 1. In Group C, inflammatory cell infiltration mainly composed of macrophages, neutrophils and lymphocytes could be seen in lung tissue sections, and congestion with hemorrhage could be seen in blood vessels. The results also showed that there were obvious inflammatory pathological changes in lung tissue of Group C. There was no obvious pathological change in Group A and B.

3.3 Pulmonary neutrophils immunohistochemistry

Figure 2 showed that there was no significant difference in the level of neutrophils in the lung tissue immunohistochemistry stain between Group A and Group B, and the level of neutrophils in Group C was significantly higher than that of Group B and Group A (green fluorescence). Figure 3 showed the level of neutrophils of the three groups. The data was expressed by the mean ± standard deviation (s). It could be seen that the level of neutrophils in the Group C was significantly higher than that of Group A and Group B ($P2 < 0.001$, $P3 < 0.001$), and there was no significant difference between Group A and Group B ($P1 = 0.9071 > 0.05$).

3.4 Mortality and infection rate of mice

Table 2 showed that there was no natural death in Group A, B and C. The results showed that mice without pneumonia after lung dissection in Group A and B, but there were 8 mice with infection in Group C and no natural death.

4 Discussions

Pneumonia, as one of the most common clinical diseases, can further lead to respiratory failure, acute respiratory distress syndrome and other diseases (Rice et al., 2012), resulting in a higher mortality. Especially for people with low immunity,

Table 1. Percentage of neutrophils of three groups of mice.

Groups	Percentage of neutrophils ($\times 10^{-2}$)
Group A	17.70 ± 3.54
Group B	16.50 ± 2.21
Group C	64.30 ± 6.15

$P_1 < 0.001$(Group B vs. Group C); $P_2 = 1.00 > 0.05$ (Group A vs. Group B); $P_3 < 0.001$(Group A vs.Group C).

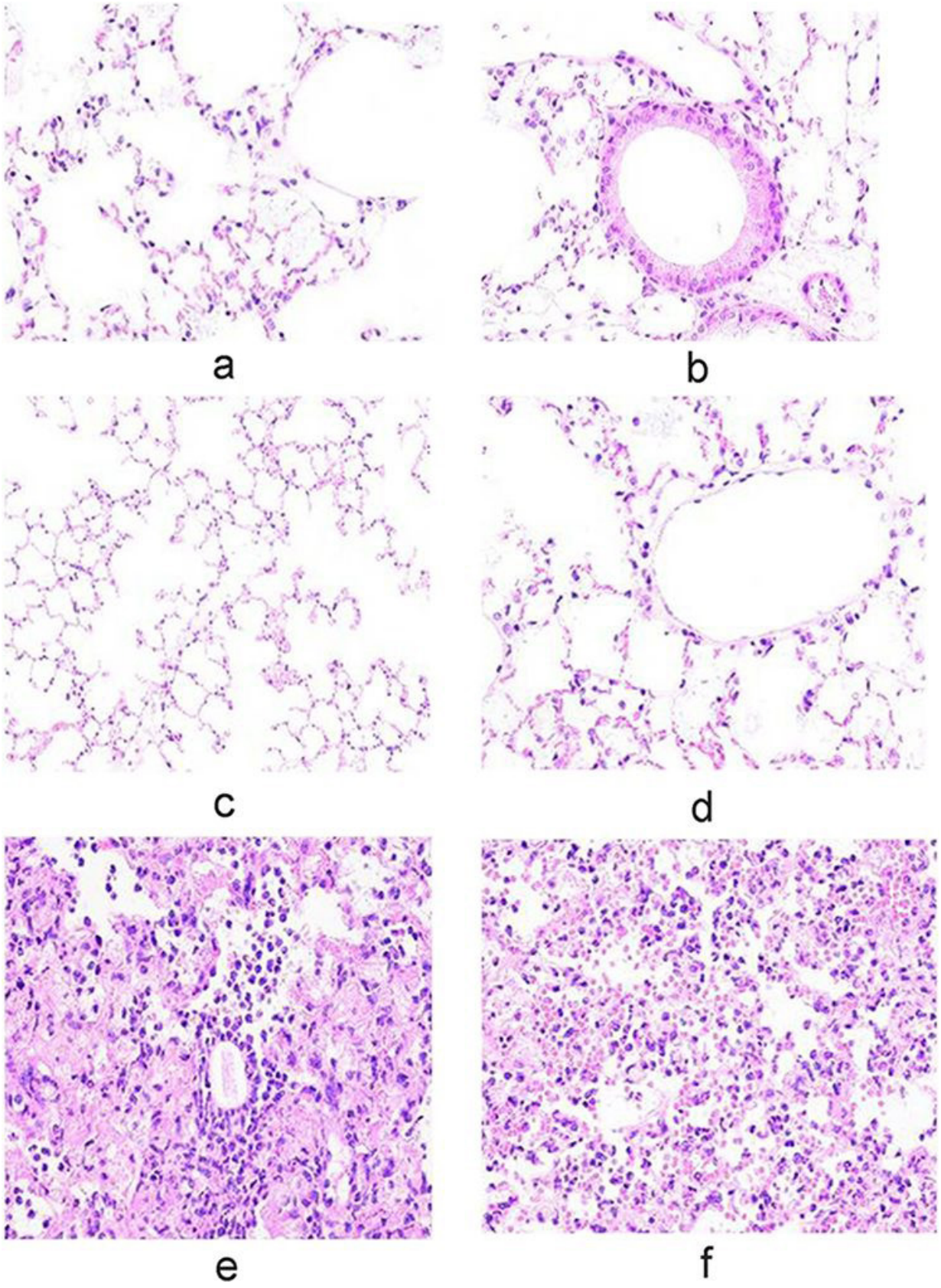

Figure 1. Pathological changes of different lung tissues in three groups of animals. (a), (b): The pathological section of Group A (200×); (c), (d): The pathological section of Group B (200×); (e), (f): The pathological section of Group C (200×); there was no inflammatory cell infiltration in Group A and Group B, and the alveolar structure was integrity. In group C, a large number of inflammatory cells exuded and alveolar structure disappeared.

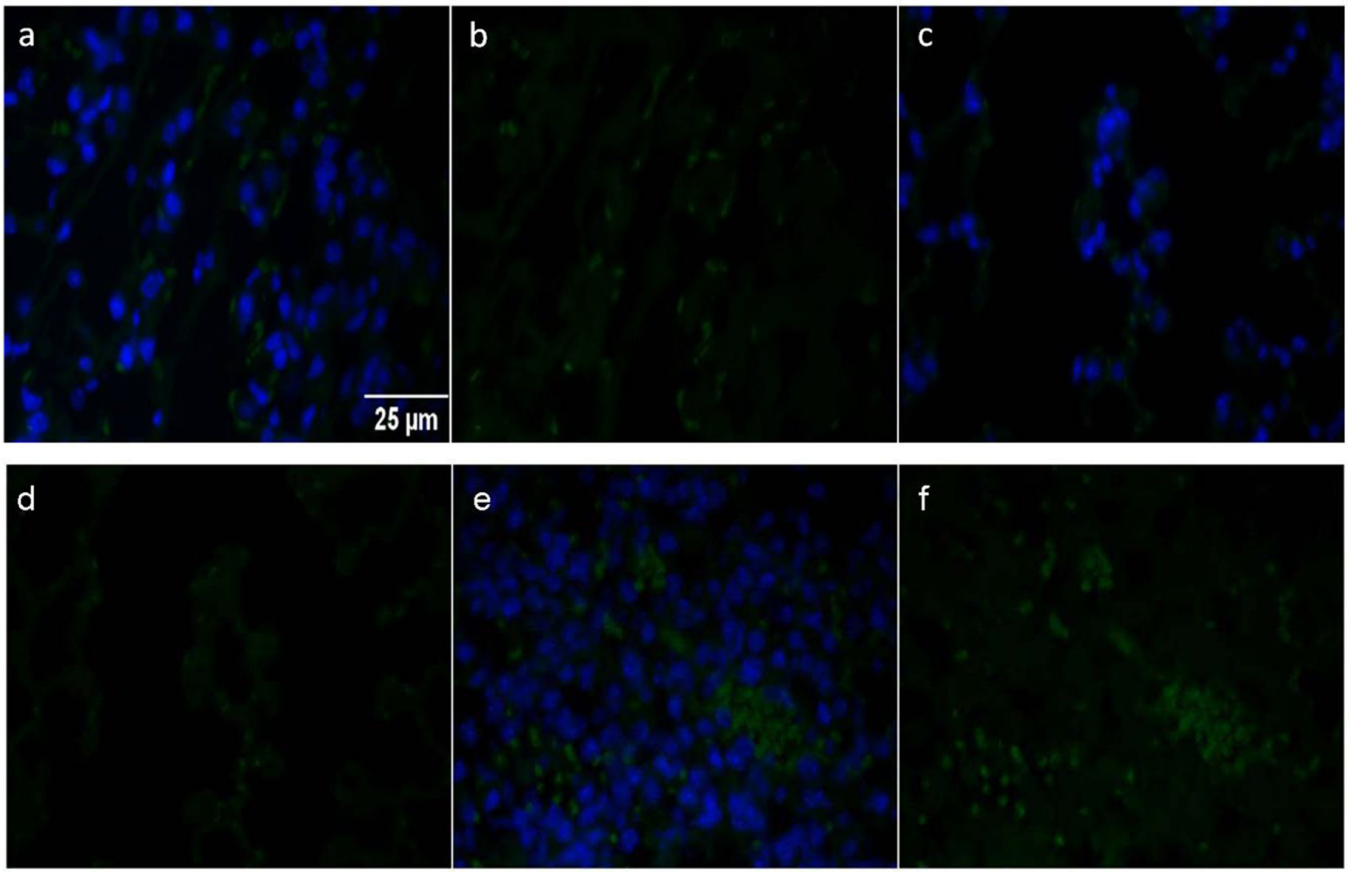

Figure 2. The immunohistochemical diagram of neutrophils in lung tissue of mice in each group (400×). [(a), (b): Group A; (c), (d): Group B; (e), (f): Group C]. Ly6g was mainly expressed in neutrophils, and we chose ly6g antibody as the first antibody and DAPI for nuclear staining. In the Figure 2, green was neutrophils and blue was other cells. Neutrophils were very rare and scattered in Group A and B. In group C, neutrophils in lung tissues were significantly more than that of Group A and B.

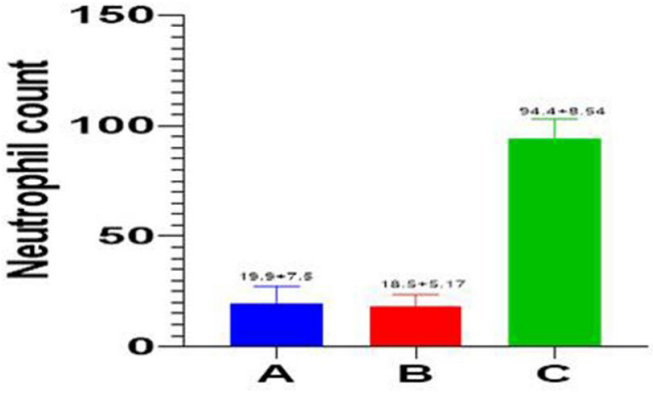

Figure 3. The number of neutrophils was obtained and analysis by one-way ANOVA. Comparison between Group A and Group B: P1 = 0.9017 > 0.05; Comparison between Group A and Group C: P2 < 0.001; Comparison between Group B and Group C: P3 < 0.001. The number of neutrophils in Group C was significantly higher than that of Group A and B, and had the statistical significance.

severe pneumonia is one of the most common lethal factors. Epidemiological results show that the probability of respiratory infection is generally increased in winter. Although the relationship between the susceptibility of respiratory diseases and

Table 2. Mortality and infection rate of mice.

Group	Infection Mice	Infection Rate (%)	Death Mice	Death Rate(%)
Group A	0	0	0	0
Group B	0	0	0	0
Group C	8	80	0	0

cold environment is generally accepted by the public, the cold environment is only recognized as an inducer (Franchini et al., 2016), which can aggravate lung inflammation, and does not have the establishment of cold stimulated pneumonia model in winter. Clinically, it can be seen that the number of patients with pneumonia or other respiratory system inflammation caused by the change of temperature difference in winter is significantly increased. In recent years, it has been reported that cold environment stimulates the immune system to reduce immunity and increase respiratory diseases. It has also been reported that cold environment directly affects the human system and affects the lung function, thus increasing the patients with pneumonia. However, due to the lack of animal models of pneumonia under cold stimulation environment, it is impossible to further study the mechanism of human pneumonia caused by the change of temperature. Because mice are susceptible to a

variety of pathogens, which are often used to establish infection models. In order to research the hypothesis that cold stimulation alone can cause pneumonia in animal model, the normal mice were exposed to low temperature repeatedly for a long time to establish a mouse pneumonia model successfully.

Figure 1 showed that there was no significant statistical difference in the percentage of neutrophils between Group A and Group B, excluding the interference of feeding environment and mice themselves on the experimental results. There was a significant statistical difference in the percentage of neutrophils in the blood of Group B and Group C ($P < 0.05$), so it could be illustrated that the percentage of neutrophils in the blood of Group C was significantly increased, and neutrophils were considered to play an important role in the progress of pneumonia (Jondle et al., 2018), which suggested that Group C had got pneumonia. Lung pathology detection showed that there was no obvious change in Group A and Group B, but a large number of inflammatory cells infiltrated into the lungs of Group C mice, the normal alveolar structure disappeared, with bronchi bleeding. There was no obvious aggregation of neutrophils in Group A and Group B, but in Group C, the number of neutrophils increased significantly compared with Group A and Group B ($P < 0.05$). Combined with the percentage of neutrophils in the blood, we could see that the mice in Group C developed pneumonia after long-term repeated exposure to cold environment, and successfully established the pneumonia model. It was suggested that pneumonia could occur directly in normal adult mice under cold environment, rather than only as an inducer to aggravate lung inflammation.

Frequent and severe changes of temperature and environment can lead to respiratory diseases such as the common cold and pneumonia. In winter, the incidence of pneumonia increased significantly (Chen et al., 2019; Ruchiraset & Tantrakarnapa, 2018). When the human body is exposed to the special environment, it reacts to the changes by adjusting its physiological functions. Cold and heat alternation caused by cold environment is one of the most common causes of pneumonia, so it is very important to further understand the relationship between temperature change and pneumonia and clarify the mechanism of the disease. However, due to the lack of appropriate animal models, the mechanism is not fully investigated. Thus, it is impossible to develop more effective methods to reduce the probability of pneumonia caused by temperature changes. Clinically, it is very necessary to prevent the deterioration progression of pneumonia caused by the change of temperature. Therefore, this study simulated the normal winter living environment of human body, exposed mice to cold environment repeatedly for a long time, and successfully established the mouse pneumonia model under cold stimulation, which provided the basis theory and the animal model for further research on the relationship between cold environment and pneumonia, and provided an effective direction for subsequent research to reduce the incidence of pneumonia caused by cold environment.

Conflict of interest

The author declare that they have no conflict of interest.

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