
Regular Article

Dynamic characteristics of sequential acute exacerbations and risk windows in AECOPD rats induced by cigarette-smoke and exposure to Klebsiella pneumoniae

Jiansheng Li\textsuperscript{a,b,c,* †}, Ya Li\textsuperscript{a,c,d †}, Xiaofan Lu\textsuperscript{a,e}, Haifeng Wang\textsuperscript{a,f}, Yang Wang\textsuperscript{a,f}, Hangjie Li\textsuperscript{a,g}, Zhaohuan Wu\textsuperscript{a},

\textsuperscript{a}Collaborative Innovation Center for Respiratory Diseases Diagnostics, Treatment and New Drug Research and Development of Henan University of Traditional Chinese Medicine (TCM), Zhengzhou, Henan 450046, China.

\textsuperscript{b}Institute for Geriatrics, Henan University of Traditional Chinese Medicine (TCM), Zhengzhou, Henan 450046, China.

\textsuperscript{c}Institute for Respiratory Diseases and the Level Three Laboratory of Respiration Pharmacology of TCM, the First Affiliated Hospital, Henan University of TCM, Zhengzhou, Henan 450000, China.

\textsuperscript{d}Central Laboratory, the First Affiliated Hospital, Henan University of TCM, Zhengzhou, Henan 450000, China.

\textsuperscript{e}Respiratory Department, The Second Clinical Medical College, Henan University of Chinese Medicine. 6 Dongfeng Road, Zhengzhou, Henan 450002, China.

\textsuperscript{f}Department of Respiratory Diseases, the First Affiliated Hospital of Henan University of TCM, Zhengzhou, Henan 450000, China.
Department of Respiratory Diseases, the Chinese Medicine Hospital of Xuchang, 19 Forward Road, Xuchang, Henan 461000, China.

† Equal contributors.

* Corresponding author: Jiansheng Li, M.D., Ph.D., Henan University of Traditional Chinese Medicine, Longzihu University Park, Zhengdong New District, Zhengzhou, Henan 450046, E-mail: li_js8@163.com.
SUMMARY

The risk-window (RW) of chronic obstructive pulmonary disease (COPD) is a period after an acute exacerbation (AE) but before the following stable phase, in which exacerbations are easy to relapse. We established a sequential COPD-AE-RW rat model by cigarette-smoke and bacterial exposures in the first 8 weeks, and was challenged with *Klebsiella pneumoniae* to mimic an AE on Day 1 of week 9, and found that body temperature, white blood cell, neutrophils, serum amyloid A (SAA) and C-reactive protein (CRP) increased in AECOPD rats 24 hours after challenge, and declined in 3 ~ 6 days, while lung function declined in 48 hours, and recovered in 7 ~ 16 days. When sacrificed, pulmonary FEV100 and FEV300 decreased, while elevated BALF neutrophils and marked airway inflammation, remodeling and emphysema were observed. Sequential COPD-AE-RW rat model was established successfully and AE phase lasts for approximately 5 ~ 7 days, followed by a 10-day around risk-window.

**Keywords**: chronic obstructive pulmonary disease, acute exacerbation, risk window, rat model, inflammation
BACKGROUND

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory respiratory disease that manifested mainly as persistent airway obstruction and aperiodic acute exacerbations (AEs).\(^1,2\) An exacerbation in COPD is an acute event that accelerates the progress of the disease, which is characterized by a worsening of the patient’s respiratory symptoms beyond normal daily variations. These events result in a change in medication and a rise in mortality.\(^3,4\)

The AECOPD risk window (RW) is an emerging concept that encompasses the period of approximately 7 to 21 days after an AE event before the patient fully returns to baseline in the stable phase. The stable phase is characterized by a relief of major symptoms without a full recovery of pulmonary function and the presence of pulmonary and systemic inflammation. Patients in this phase are at increased risk of experiencing another exacerbation, and sequential treatments for AE-RW have been effective.\(^5\) Although few studies have examined the extended period that follows a COPD exacerbation, there is some evidence showing that there is always an unstable condition after the exacerbation phase but before stable phase: more than 50\% of COPD patients who suffered an AECOPD event experience a period where they are at high-risk of recurrence during the 8 weeks following the first exacerbation.\(^6,7\) In COPD, the recovery of pulmonary function after an exacerbation may require more than 1 month, and the more frequent the exacerbations, the longer the recovery time.\(^8,9\) Published data have shown that indicators of airway inflammatory were recovered in lung lavage fluids at 4 days after an acute exacerbation in AECOPD patients, while biomarkers of systemic inflammation had not returned to baseline levels within 14 days.\(^10\)
To observe the dynamic characteristics of the AE-RW period, indicators were selected based on their sensitivity to inflammation. Frequent acute exacerbations can be triggered by viral upper respiratory tract infections and/or bacterial infections, and these events aggravate pulmonary inflammatory reactions. Bronchoscopic examinations indicated that at least 78% of patients with exacerbations have a viral and/or bacterial infection: viruses were found in 48.4% (6.2% when stable) and bacteria in 54.7% (37.5% when stable). During an inflammatory response to an infection, the number of neutrophils in patients’ sputum and blood increased during exacerbations and was positively associated with disease severity. C-reactive protein (CRP) and neutrophils, the most sensitive indicators of inflammation, were significantly higher during the AE phase of COPD and depressed during the stable phase.

*Klebsiella pneumoniae*, which is one of top three pathogens causing COPD deterioration and has been used to prepare animal models of COPD at the stable stage and the acute exacerbation stage. In this study, we used rat model of COPD and AECOPD that induces significantly lower pulmonary function, especially in acute inflammatory reactions, to explore dynamic inflammatory features during and after an acute bacterial challenge with *Klebsiella pneumoniae* in a COPD rat model. The rats were challenged with an additional exacerbation at a different time-point to determine variations in the temporal course of AE and the subsequent RW.

**METHODS**

**Animals**

Twenty male and 20 female Sprague-Dawley rats that were 2 months old and weighed 200 ± 20 grams (g) were obtained from the Experimental Animal Center of Henan Province...
Biological and Pharmaceutical Bulletin Advance Publication

(Special Pathogen Free level, Certificate: SCXK (Henan) 2005-0001) and housed in individual ventilated cases for seven days before the experiments in a facility in the First Affiliated Hospital, Henan University of Traditional Medicine, Zhengzhou, Henan, China. Room temperature was maintained at (25 ± 1) °C, relative humidity at (50 ± 10) %, with 10 to 15 gas changes per hour, an ammonia concentration $\leq 14 \text{ mg/m}^3$, and noise $\leq 60 \text{ db}$. A sterilized diet and water were freely available.

**Bacteria**

*Klebsiella pneumoniae* (KP; strain: 46114) was provided by the National Center For Medical Culture Collections (Beijing, China). The cultures were prepared at concentrations of suspensions of $6 \times 10^8$ and $6 \times 10^{14}$ colony-forming units (CFU) before challenge.

**Cigarettes**

Hongqi Canal® Filter cigarettes (tobacco type, tar 10 mg; nicotine content 1.0 mg; and carbon monoxide, 11 mg) were provided by the Henan Zhongyan industrial company (Zhengzhou, Henan).

**Grouping and model preparation**

Forty rats were randomly separated into Control, COPD, AE1 and AE2 groups using a random numbers table. Five male and 5 female rats were assigned to each group. After the rats were adaptive accommodated for 7 days, COPD was induced via exposure to cigarette smoke (the particulate concentration was maintained at 3000 ± 500 parts per million during two 30-min exposure periods per day on Monday through Saturday) and nasal inhalations of a KP solution ($6 \times 10^8$ CFU per mL, 0.1 mL, every 5 days) while the animals were in a waking state for 8 weeks. To mimic an AE, orotracheal intubation injections containing KP
(6 × 10^{14} \text{ CFU per mL, 0.1 mL}) were used to challenge the rats after they were anesthetized using 10% chloral hydrate at a dose of 2.8 mL/kg body weight.\textsuperscript{19,26,27} In the AE1 group, the challenges were performed on Day 1 and Day 15, which were counted from the first day of week 9; and in the AE2 group, the challenges were performed on Day 1 and Day 23.

**General status**

Body weights were recorded weekly throughout the entire experiment, and body temperatures were tested every 2 days from Day 0 through Day 44.

**Pulmonary function tests**

Pulmonary function was measured using an unrestrained whole body plethysmography (WBP) system (Buxco, NY, USA) every weekend and every 24 h after bacterial challenge. To measure pulmonary function, the rats were placed in a sealed box that was connected to transducers and a computer, and tidal volume (V\textsubscript{T}), peak expiratory flow (PEF) and 50% tidal volume expiratory flow (EF50) were calculated. To detect forced vital capacity (FVC) and forced expiratory volume at 300 milliseconds (FEV0.3), the rats were anesthetized on Day 44, and then an endotracheal intubation with tracheotomy was performed, and the rats were tested using a FinePointe™ Pulmonary Function Test (PFT) system (Buxco, NY, USA).

**Peripheral blood cytological and acute inflammatory analysis**

From Day 0 to Day 44, 0.4 mL of tail vein blood was sampled every 2 days. Cytological analyses were performed using 0.2 mL of blood to determine characteristics including the numbers of white blood cells (WBCs), neutrophils, lymphocytes and monocytes using a haemocyte analyser. The remaining 0.2 mL of blood was centrifuged to determine
serum levels of C-reactive protein (CRP) and amyloid A (SAA) using enzyme-linked immunosorbent assays (ELISA) (Boster, Wuhan, China).

**Lung tissue sectioning and bronchoalveolar lavage**

At the time of sacrifice, the animals were deeply anesthetized, and 8 ml of whole blood was collected from the aorta abdominalis. The animal was then exsanguinated. The trachea was cannulated, and the heart/lung block was removed from the thoracic cavity. The right extrapulmonary bronchus was ligated using a suture, and the right lung lobes were removed. The left lung lobe was lavaged with saline, and the lavage fluids (bronchoalveolar lavage fluid; BALF) that were recovered from each rat were analysed to determine total and differential cell counts. After bronchoalveolar lavage was performed, the left lung lobe was perfusion-fixed via the trachea by pushing 10% neutral buffered formalin through the tissue at a constant pressure of 30 cm of fixative. After 2 h of intratracheal fixation, the trachea was ligated, and the lung lobe was immersed in a large volume of the same fixative for at least 24 h before it was further processed for light microscopy, as described below.

**Analysis of BALF**

Total cells were recovered from bronchoalveolar lavages, and cell counts were manually determined using a haemocytometer. Differential cell counts (e.g., neutrophils, macrophages, lymphocytes) were determined by counting 200 cells per animal.

**Pulmonary morphology and morphometry**

Randomly orientated serial sections of formalin-fixed left lung lobe tissue were processed using routine methods and then embedded in paraffin. Tissue sections (4 μm thick) were deparaaffinized and stained with hematoxylin and eosin (H&E) for histopathology. The
observers who evaluated the slides were blinded, and the alveolar cavity and the densities of
the alveoli were determined as follows: mean linear intercept (MLI) (μm) = L/Ns. After a
cross (+) was drawn through the centre of each photo, the number of alveolar septa (Ns) that
were under the cross was counted, and then the total length of the cross (L) was measured.
The following equation was used to determine the number of pulmonary alveoli in each
visual field (Na): mean alveolar numbers (MAN) (/mm2) = Na/A, and the area of the visual
field (A) was measured.29)

**Statistical analysis**

The data are presented as the mean ± standard error (SE). For mortality-related data,
chi-square tests were used to detect differences between groups. For repeated measures data,
such as body weight, body temperature, pulmonary function, cytology and inflammatory
indexes, a general linear regression equation was used to detect differences in overall group
effects. For one-time measurement data and intergroup comparisons of repeated measures at
each time point, one-way ANOVA was used to detect differences between groups. All
statistical analyses were performed using SPSS Statistics 19.0 (IBM, CA, USA), and \( P<0.05 \)
was set as the threshold for statistical significance.

**RESULTS**

**Mortality**

At 24 h after the first bacteria challenge, 1 rat in the AE1 group and 1 rat in the AE2
group died as a result of a pulmonary abscess, and another rat died at 24 h after the second
bacterial challenge in the AE2 group. The total mortality rates were 0%, 0%, 20% and 10% in
the Control, COPD, AE1 and AE2 groups, respectively (Table 1).

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**Body weight**

The COPD rats showed a slightly slower gain in body weight than the Control group during the first 8 weeks, especially in week 8 ($P<0.01$), and this difference became wider after week 8. After the second challenge on the first day of week 11 (Day 15), body weight gains were significantly lower in the AE1 group from week 11 through week 14 ($P<0.05$) (Fig. 1A). Body weight also showed a downward trend in the AE2 group from week 12 to week 14 ($P<0.05$), after the second bacterial infection, but weight gain remained significantly higher in the AE2 group than in the AE1 group in weeks 13 and 14 ($P<0.05$). Overall, body weight gain was lower in the COPD group than in the Control group during first 8 weeks ($P<0.05$), and it decreased significantly more rapidly in the AE-RW period ($P<0.05$), especially in AE1 group ($P<0.05$) (Fig. 1 B and C).

**Body temperature**

As shown in Fig. 1D, there was no statistical difference in body temperature between the COPD and Control groups. Body temperature were higher in the AECOPD groups for 24 h post-bacterial challenge than temperatures in the COPD group at each time point ($P<0.05$). In the challenged animals, temperatures quickly decreased over the following 4 days but remained higher on Day 8 ($P<0.05$). From Day 10 up to Day 14, body temperatures in the AECOPD groups were similar to those in the COPD group, and they were synchronized after Day 14. When the second bacterial challenge was performed on Day 15, a much higher elevation in body temperature was observed in the AE1 group on Day 16 than was observed on Day 2 ($P=0.001$), and the recovery time was 2 days longer than the time for required after the first challenge. However, a similar curve was followed, and the challenged groups were
synchronized with the COPD group on Day 34. When the second challenge was performed on Day 22 in the AE2 group, the higher curve and range for body temperature were similar to those that were observed after the first exacerbation, but they were significantly lower on Day 24 than the values observed in the AE1 group on Day 16 ($P=0.000$). In addition, the AE1 group had a recovery period that was 2 days shorter.

**Pulmonary function**

During the 14-week experimental period, the tendency of $V_T$, PEF and EF50 to vary in the COPD group was significantly lower than in the control group ($P<0.05$) (Fig. 2A, B and C). In COPD rats, $V_T$, PEF and EF50 gradually decreased during the first 8 weeks, and they were stable from week 8 to week 14 ($P<0.05$). After the animals were challenged with KP solution on the first day of week 9 (Day 1), $V_T$, PEF and EF50 were lower in the AECOPD rats than in the COPD rats within 24 hours ($P<0.05$), and this was followed by a gradual recovery over the next 2 weeks, with $V_T$ and EF50 remaining lower in the AECOPD group than in the COPD group in week 9 ($P<0.05$). The values obtained for the indicators in the AECOPD groups were close to the values in the COPD group in week 10, and $V_T$ and PEF were also similar between the AE2 group and the COPD group in week 11. In the AE1 group, after the second challenge on the first day of week 10, $V_T$, PEF and EF50 rapidly decreased over 24 h until they fell in a smaller range on Day 2 ($P=0.001$) than in either the COPD or the AE2 group ($P<0.05$). However, they were higher than the values in the COPD group on week 11 ($P<0.05$), and they then recovered quickly during this first week post-challenge and gradually increased over the following 2 weeks until the values were similar to those in the COPD group in week 13. There was also a sharp decline in $V_T$, PEF
and EF50 at 24 h after the second challenge in the AE2 group \( (P<0.05) \), which demonstrated a smaller peak value than was observed in the AE1 group on Day 16 \( (P=0.001) \). In the AE2 group, \( V_T \), PEF and EF50 recovered quickly during the first week and then increased gradually over the following days until they had synchronized with the COPD group values in week 14.

As shown in Fig. 2D, FVC, FEV0.3 and FEV0.3/FVC were lower in the COPD group than in the Control group \( (P<0.05) \), and they were significantly lower in the AECOPD groups than in the COPD group \( (P<0.05) \), with the AE1 rats displaying even lower values \( (P<0.05) \).

**Cellular categories in peripheral blood**

As shown in Fig. 3, during the AE-RW-COPD period, the number of WBCs was higher in the COPD group than in the Control group from Day 0 to Day 4, and the numbers of neutrophils and monocytes were higher from Day 0 to Day 8 \( (P<0.05) \). After the first challenge with KP solution on Day 1, the numbers of WBCs, neutrophils and monocytes were significantly higher in the AECOPD groups than in the COPD group on Day 2 \( (P<0.05) \). WBCs then quickly decreased in numbers, with a significantly higher number observed in the AE groups than in the COPD group for the following 6 days \( (P<0.05) \). Neutrophils and monocyte remained significantly higher for 4 days \( (P<0.05) \). All counts returned to the levels observed in the COPD group by Day 16 to 18, indicating that this process takes about 10 to 12 days. In the AE1 group, the second challenge was also followed by highly elevated numbers of WBCs, neutrophils and monocytes after 24 h \( (P<0.05) \). There was then a rapid decrease in WBC and neutrophil counts from Day 18 to Day 22 and in monocyte counts from
Day 18 to 20, compared to the trend observed in the COPD and AE2 groups \( (P < 0.05) \). Counts then smoothly declined over the next 8 to 10 days until they were merged across groups on Day 30. In the AE2 group, after the second challenge with KP solution on Day 23 in the AE2 group, WBC, neutrophil and monocyte counts were higher after 24 hours in the AE2 group than in the COPD and AE1 groups \( (P < 0.05) \), and WBC counts rapidly declined over the following 6 days, while neutrophil and monocyte counts declined over the next 4 days. The counts returned to baseline levels that were similar to those observed in the COPD group over the following 10 to 12 days. During the second challenge, the numbers of WBC and neutrophils in the AE2 group were higher than the values in the COPD and AE1 groups from Day 24 to Day 30, while monocyte counts were higher on Day 24 and Day 26 \( (P < 0.05) \). The elevation in the range of values observed for WBC, neutrophil and monocyte counts after the second bacterial challenge in the AE1 group on Day 16 was higher than the increase observed on Day 2 \( (P = 0.002, P = 0.024, \text{ and } P = 0.028, \text{ respectively}) \), and the range of WBC and neutrophil counts was also higher than the values observed on Day 24 \( (P = 0.002 \text{ and } P = 0.017, \text{ respectively}) \). This variation in the trends associated with lymphocyte counts across the 4 groups did not display any clear regularity, and no significant differences were discovered.

**Cellular categories in bronchial alveolar lavage fluid**

At the end of experiment, the numbers of neutrophils, macrophages and lymphocytes were significantly higher in the COPD group than the Control \( (P < 0.05) \) (Fig. 4A, B and C). The numbers of neutrophils and macrophages in the AE1 and AE2 groups were significantly higher at the end of the experiment than in the COPD group \( (P < 0.05) \), while lymphocyte
numbers did not significantly changed.

**C-reactive protein and serum amyloid A levels in serum**

From Day 0 through Day 44, which encompass the AE-RW-COPD period, CRP and SAA levels were significantly higher in the COPD group than in the Control group ($P<0.05$) (Fig. 5). CRP and SAA levels were elevated at 24 h after bacterial challenge, and they then quickly and significantly declined during the following 4 - 6 days compared to levels lower than those observed in the COPD group ($P<0.05$). They then gradually decreased until they reached baseline levels similar to those in the COPD group on Day 16. In the AE1 group, CRP and SAA levels increased to higher levels on Day 2, at 24 h after the second bacterial challenge ($P=0.048$, $P=0.019$), and then rapidly declined over the following 4 to 6 days. The AE1 values were significantly different from those in the COPD and AE2 groups ($P<0.05$). Over the following 10 to 12 days, the levels gradually decreased until they became similar to those in the COPD group on Day 32. In the AE2 group, the increase observed following the second bacterial challenge was lower than the increase observed in the AE1 group on Day 16 ($P=0.018$, $P=0.009$), and they sharply declined but remained significantly higher than the values in the COPD and AE1 groups over the next 4 to 6 days ($P<0.05$). Finally, in the AE2 group, CRP and SAA levels were synchronized with the levels in the COPD group on Day 40 and Day 38, while CRP levels remained higher than in the COPD and AE1 groups on Days 30 and 32, and SAA levels remained higher on Day 30 ($P<0.05$).

**Lung morphology and morphometry**

As shown in Fig. 6A, B and C, no obvious pathological impairment was observed in the Control group. Marked chronic bronchiolar and pulmonary obstruction, airway wall...
thickening and hyperplasia, and alveolar destruction were observed in the COPD rats (Fig. 6D, E), and obvious inflammatory cell infiltration was also found, as shown in in Fig. 6F. In the rats suffering from AE, the airway walls were thickened and hyperplastic, and alveolar destruction and observable inflammatory cell aggregation were observed, especially the AE1 group (Fig. 6G to L).

As shown in Fig. 6M and N, MLI was much higher in the COPD group than in the Control group, and MLI was even higher in the AE groups than in the COPD group. However, MLI was lower in the AE2 than in the AE1 group \((P<0.05)\), whereas MAN was lower in the COPD group than in the Control group and lower in the AE groups, especially the AE1 group, than in the COPD group \((P<0.05)\).

**DISCUSSION**

This is the first study to describe the dynamic inflammatory features of a COPD-AE-RW rat model induced by using cigarette-smoke and bacterial exposures. Data indicate that infectious challenged in the risk-window can initiate additional and more severe inflammatory response and pulmonary impairment than those happened in the stable phase.

COPD was characterized by chronic and recurrent inflammation response in lungs and airway, which might take years or decades development. Cigarette smoke and bacterial/viral infections often trigger inflammatory responses in the airway epithelium and alveoli,\(^{30,31}\) where they initiate chronic bronchitis, airway remodelling and alveolar destruction, resulting in a decline in pulmonary function.\(^{32,33}\) Therefore, we chose CS exposures and *Klebsiella pneumoniae* infections to established a rat model to evaluate the features of COPD and for further pharmacological study.
The risk-window is a period after the AE but before an individual return to stable status. This window lasts for approximately 7 to 21 days in human clinical studies, and in which the sequential medication applied to AE-RW patients are believed to accelerate the recovery course and prevent another exacerbation.\textsuperscript{14,17,34} In this study, it was observed that AE period was about 5 to 7 days long, which is varied according to sharp differences in inflammatory indicators. Then we challenged the AECOPD rats again at different time-points, separately, which were Day 15, the day 7 days after the first AE phase, falling in the RW, and Day 23, the day 15 days after the first AE phase, falling outside of the risk window. The selection was based on the suggested application of 5 to 10 days of antibiotic medication in \textit{“Global Initiative for Chronic Obstructive Lung Disease”} (GOLD).

Pulmonary function and serum inflammatory response proteins are widely used as representative biomarkers reflecting the occurrence and status of exacerbation.\textsuperscript{35-38} CRP and SAA irregularly paralleled the tendency toward variation that was observed in the populations of WBCs and neutrophils.\textsuperscript{39-43} In this study, pulmonary function were significantly lower in AECOPD rats 24 h after the first bacterial challenge and did not fully recover in 2 weeks, and SAA, CRP rapidly increased 24 h after challenge and then quickly decreased in 4 ~ 6 days, but not fully recover to stable phase. During the following 10 to 12 days, SAA and CRP fluctuated slightly but not approached the baseline of COPD rats, which might indicate that the rats are at the RW phase. Days after Day 13 could be considered as the start of the following stable phase of COPD. Similar variation trends and time-courses were observed in the changes of populations of white blood cells, neutrophils and monocytes.

The data also showed that the second exacerbation happened in RW phase (Day 15)
induced lower FVC and FEV0.3, and more severe inflammatory response than the first challenge (Day 1), such as 2 ~ 4 days delayed elevated neutrophils and CRP. On the contrary, the AE induced in stable phase (Day23) showed similar variation to challenge 1, in the extent of airway and systemic inflammation and duration. These results indicated that exacerbation happened in RW might be more severe than that happened in stable phase and lasts longer.

In the pathogenesis of COPD, recurrent inflammation, airway injury and repairs always lead to airway remodelling and further progression of disease, especially in the presence of frequent acute exacerbations.44, 45) In this study, we observed that structural abnormalities in small conducting airways and adjacent alveoli were markedly increased in both AECOPD and COPD lungs and were more severe in AECOPD rats when characteristics such as the infiltration of inflammatory cells, excessive mucus secretion, alveolar destruction and small airway remodelling were compared. Emphysema in AECOPD rats, and these changes were much more severe in the AE1 group than in the AE2 group. Additionally, we found that the above-mentioned pathological changes were more severe in the AE1 rats than that in the AE2 rats that were challenged after a longer interval of time.

It was previously reported that the RW lasts for about 7 to 21 days in COPD patients, based on observations of the recovery of pulmonary function and inflammatory responses.17) However, few details were available regarding the time course of airway and systemic inflammatory responses during the AE-RW phases in animal models.28,46-48) To obtain a definition of the AE and RW periods in a rat model of COPD, we observed the dynamics of variations in the levels of inflammatory indicators for 43 days after the first bacterial challenge was administered, and we found that systemic inflammatory indicators, such as
those obtained in routine blood cell analyses in addition to serum CRP and SAA levels, recovered quickly within 5 to 7 days after the bacterial challenge and then switched to a slower recovery period that lasted for approximately 11 to 13 days (Table 2).

Our study has a limitation. The limitation to be considered is the fact that we did not study the microbial colonization or the colonization level on the respiratory tract in this study. Previously, we studied the bacterial infection induced COPD model compared with cigarette-smoke, and the combination of the two, and it did produce COPD-like impairments in the airways and lungs \(^{26}\). Therefore, we believe the method of the model building were mature and reproducible. We have initiated another specific study about microorganisms colonize on the pulmonary and intestinal flora in COPD in our laboratory to cover the limitation in this study.

CONCLUSION

Sequential variation was successfully observed in AEs and RWs in a rat model of COPD that was induced using cigarette-smoke and exposure to bacterial infection. In these animals, the AE phase lasted for approximately 5 to 7 days and was followed by an approximately 11 to 13 day RW.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The study protocol was approved by the Ethics Committee of The First Affiliated Hospital, Henan University of Traditional Medicine, Zhengzhou, Henan, China. And the study was indeed conducted in accordance with the guidelines.
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**Figure 1.** Temporal changes in body weight and body temperature. A: changes in body weight in 14-week; B and C: body weight gain in the COPD preparation and AE-RW-COPD period; D: changes in body temperature from Day 0 to Day 44 in each group. Repetitive measurement deviation analysis in body weight and temperature: \( P = 0.000 \). AE: acute exacerbation; COPD: chronic obstructive pulmonary disease; RW: risk window. Sample size: \( N = 8 \). *\( P < 0.05 \), COPD vs. Control group; ▲\( P < 0.05 \), AE1 vs. COPD group; #\( P < 0.05 \), AE2 vs. COPD group; ●\( P < 0.05 \), AE2 vs. AE1 group. Bacteria challenge was performed on Day1, Day15 and Day23. (Color figure can be accessed in the online version.)

**Figure 2.** Temporal changes of parameters of lung function. Parameters tested during the 14-week experiment period: (A) tidal volume (\( V_t \)), (B) peak expiratory flow (PEF), (C) expiratory flow at 50% (EF50). Parameters tested on Day 44: (D) forced vital capacity (FVC), (E) forced expiratory volume 0.3 s (FEV0.3), (F) FEV0.3/FVC. Repetitive measurement deviation analysis in \( V_t \), PEF and EF50: \( P = 0.000 \). AE: acute exacerbation; COPD: chronic obstructive pulmonary disease. Sample size: \( N = 8 \). *\( P < 0.05 \), COPD vs. Control group; ▲\( P < 0.05 \), AE1 vs. COPD group; #\( P < 0.05 \), AE2 vs. COPD group; ●\( P < 0.05 \), AE2 vs. AE1 group. Bacteria challenge was performed on the first day of week 9 (Day 1) and week 11 (Day 15), and the second day of week 12 (Day 23). (Color figure can be accessed in the online version.)

**Figure 3.** Temporal changes in the population of inflammatory cells during AE-RW-COPD period. A: white blood cell; B: neutrophils; C: monocyte; D: lymphocyte. Repetitive measurement deviation analysis in WBC, neutrophils: \( P = 0.000 \), monocyte: \( P = 0.045 \); lymphocyte: \( P = 0.122 \). AE: acute exacerbation; COPD: chronic obstructive pulmonary disease, WBC: white blood cell. Sample size: \( N = 8 \). *\( P < 0.05 \), COPD vs. Control group; ▲\( P < 0.05 \), AE1 vs. COPD group; #\( P < 0.05 \), AE2 vs. COPD group; ●\( P < 0.05 \), AE2 vs. AE1 group. Bacteria
challenge was performed on Day1, Day15 and Day 23. (Color figure can be accessed in the online version.)

Figure 4. Neutrophils (A), lymphocyte (B) and macrophage (C) in bronchial alveolar lavage fluid on Day 44.

AE: acute exacerbation; BALF: bronchial alveolar lavage fluid; COPD: chronic obstructive pulmonary disease.

Sample size: N = 8. *P < 0.05, COPD vs. Control group; ▲P < 0.05, AE1 vs. COPD group; ◊P < 0.05, AE2 vs. COPD group; ●P < 0.05, AE2 vs. AE1 group. (Color figure can be accessed in the online version.)

Figure 5. Temporal changes in C-reactive protein (A) and serum amyloid A (B) during the AE-RW-COPD period. Repetitive measurement deviation analysis in CRP and SAA: P = 0.000. AE: acute exacerbation; COPD: chronic obstructive pulmonary disease; CRP: C-reactive protein; SAA: serum amyloid A. Sample size: N = 8.

*P < 0.05, COPD vs. Control group; ▲P < 0.05, AE1 vs. COPD group; ◊P < 0.05, AE2 vs. COPD group; ●P < 0.05, AE2 vs. AE1 group. Bacteria challenge was performed on Day1, Day15 and Day 23. (Color figure can be accessed in the online version.)

Figure 6. Representative pathological pictures in the lung tissues in each group. A, B and C: Control group; D, E and F: COPD group; G, H, I: AE1 group; J, K, L: AE2 group. Magnification: A, D, G and J, × 100; B, E, H and K, × 200; C, F, I and L, × 400. The changes of mean linear intercept (MLI) (M) and mean alveolar numbers (MAN) (N) in each group. AE: acute exacerbation; COPD: chronic obstructive pulmonary disease. Sample size: N = 8. *P < 0.05, COPD vs. Control group; ▲P < 0.05, AE1 vs. COPD group; ◊P < 0.05, AE2 vs. COPD group; ●P < 0.05, AE2 vs. AE1 group. (Color figure can be accessed in the online version.)
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

A

CRP in serum (pg/ml)

Time (day)

Control COPD AE1 AE2

P = 0.048

P = 0.018

P = 0.000

B

SAA in serum (pg/ml)

Time (day)

Control COPD AE1 AE2

P = 0.019

P = 0.009

P = 0.000
Figure 6
Table 1. Mortality rates in the rats in the Control, COPD and AECOPD groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Sample size</th>
<th>No. of deaths</th>
<th>Mortality (%)</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COPD</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AE1</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>AE2</td>
<td>10</td>
<td>1</td>
<td>10</td>
</tr>
</tbody>
</table>

Note: AE, acute exacerbation; COPD, chronic obstructive pulmonary disease.
<table>
<thead>
<tr>
<th>Indicators</th>
<th>Time of exacerbation (Date)</th>
<th>AE Period (day)</th>
<th>Period of AE Begin (Date)</th>
<th>RW Period (day)</th>
<th>Period of RW (Date)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
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<td>7, 23, 36, 13</td>
<td>8, 21, 13</td>
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<tr>
<td>WBC</td>
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<td>1, 8, 7</td>
<td>9, 20, 11</td>
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<tr>
<td>Neutrophils</td>
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<td>7, 18, 11</td>
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<tr>
<td>Monocyte</td>
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<tr>
<td>CRP</td>
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<td>1, 6, 5</td>
<td>7, 18, 11</td>
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<tr>
<td>SAA</td>
<td>15, 23, 15, 23</td>
<td>1, 8, 7</td>
<td>9, 20, 11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: AE, acute exacerbation; CRP, C-reactive protein; EF50, 50% tidal volume expiratory flow; PEF, peak expiratory flow; RW, risk window; SAA, serum amyloid A; V_T, tidal volume; WBC: the number of white blood cells.