

Original Article

Improvement effect of doxofylline on the mechanism of glucocorticoid resistance in chronic obstructive pulmonary disease rat model

Nengluan Xu^{1,2,3}, Yusheng Chen^{1,2,3}, Xiaoyan Chen^{1,2}, Hongru Li^{1,2}, Wensen Huang¹, Zhengwei Chen¹, Haibo Wang¹, Hongli Deng¹, Qiusheng Yang¹

¹The Shengli Clinical Medical College of Fujian Medical University, Fujian Provincial Hospital, Fujian Medical University, Fuzhou, Fujian Province, China; ²Department of Respiratory and Critical Care Medicine, Fujian Provincial Hospital, Fuzhou, Fujian Province, China; ³The Laboratory of Four Respiratory Diseases, Fujian Provincial Hospital, Fuzhou, Fujian Province, China

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Abstract: Objective: To observe whether doxofylline can enhance the sensitivity of rats with chronic obstructive pulmonary disease (COPD) to glucocorticoid by reactivating the histone deacetylase 2 (HDAC2). Methods: Thirty COPD rat models were established by cigarette exposure (CE). Twelve weeks later, six rats were randomly selected to test whether the model was established successfully with other six healthy rats serving as controls. The other twenty-four rats, which were successfully modeled, were randomly divided into cigarette exposure group (CE group), doxofylline group (taking doxofylline orally, CE+D group), budesonide group (taking budesonide by aerosol rebreathing method, CE+B group) and budesonide + doxofylline group (CE+B+D group). Then, the pathological morphology of lung tissue of rats in each group was observed. And the quantitative analysis of morphology, the count and classification of BALF inflammatory cells, the detection of the expression levels of IL-8 and TNF- α in serum and BALF supernatant and the protein expression levels of HDAC2, P-PI3K, P-Akt in lung tissue were performed. Besides, the activity of HDAC2 was measured, and the correlation between HDAC2 activity and other indexes was analyzed. Results: Compared with CE group, the pulmonary function and the degree of pulmonary damage in CE+B+D group and CE+D group were improved, the number of inflammatory cells generally decreased and the inflammatory factors decreased significantly in serum and bronchoalveolar lavage fluid. And the CE+B group showed no significant changes. These findings indicated that hormone resistance existed in rat COPD model, and doxofylline could improve the hormone resistance. Compared with CE group, the expression of HDAC2 in lung tissues was increased significantly (both $P < 0.001$) and HDAC2 activity was enhanced ($P = 0.021$, $P = 0.006$) in CE+D group and CE+B+D group while there was no significant improvement in the expression and activity determination of HDAC2 in CE+B group. In addition, the expression of P-PI3K and P-Akt in lung tissues of rats was reduced in CE+D group and CE+B+D group compared with CE group (all $P < 0.001$). Conclusion: On the basis of the use of glucocorticoid, doxofylline might improve the resistance in COPD model rats to glucocorticoid by restoring HDAC2 activity.

Keywords: Chronic obstructive pulmonary disease, rat model, resistance to glucocorticoid, doxofylline, histone deacetylase 2

Introduction

Chronic obstructive pulmonary disease (COPD) is a pulmonary disease characterized by airflow limitation, and its attack is related to the abnormal inflammatory reaction of the lungs to harmful gases such as cigarette smoke or noxious particles [1, 2]. World Health Organization estimates that COPD will rise from the fourth place to the third place in the global ranking of mortality by 2020 [3]. At present, some drugs, such

as bronchodilators, anti-inflammatory drugs and antioxidant drugs, are usually used in the clinical drug treatment of COPD [4]. In all kinds of anti-inflammatory drugs, the anti-inflammatory effect of glucocorticoid (GC) is the best, but its clinical efficacy in the treatment of COPD is not significant and cannot delay the course of disease and reduce mortality. Meanwhile, COPD patients show resistance to GC, and the more serious the disease is, the higher the degree of the GC resistance exists. Therefore, it

is urgent to study the intrinsic mechanism of GC in order to promote the clinical application of drugs with GC in the treatment of COPD.

Research has shown that the presence of GC resistance in COPD may be due to the decrease of histone deacetylase 2 (HDAC2) activity *in vivo*, whereas HDAC2 is a protease that plays an important role in the structural modification of chromosomes and the regulation of gene expression [5]. A study also has shown that COPD is mainly regulated by the expression of genes related to various inflammatory factors, such as IL-8 and TNF- α , and the increase of transcription of these confirmatory factor genes can be inhibited by HDAC2 [6].

It was reported that low-dose theophylline could inhibit the activity of PI3K directly, which led to the inactivation of the PI3K/Akt pathway, the recovery of the expression level and activation degree of HDAC2, thereby reversing the hormone resistance [7]. Compared with theophylline, doxofylline, after the structure optimization, had little side effect on the gastrointestinal tract and strong effect on bronchial dilation [8]. However, both targets were phosphodiesterase (PEN) [9]. Thus, we speculated that doxofylline also might improve the hormone resistance in patients with COPD by regulating the expression of HDAC2 through the PI3K/Akt pathway. Because of few relevant reports in the domestic and overseas, this study aimed at studying the improvement mechanism of doxofylline in the hormone resistance in COPD model based on the successful establishment of COPD rat model with the simple cigarette exposure (CE) method.

Materials and methods

Experimental animals

Thirty-six male Wistar rats, a body weight of 202 ± 5 g and an age of 8 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., and kept in SPF Animal Laboratory in the Department of Comparative Medicine of Fuzhou General Hospital of Nanjing Military Area Command of Chinese PLA. Their feed, water and bedding were strictly sterilized and they were given humane care based on the 3R principle (reducing, reusing and recycling) of animal experiment.

Reagents and instruments

The reagents and instruments were listed as follows: homemade glass exposure box, cigarettes (Hongta Tobacco Group Co., Ltd.), Ani-Res2005 animal pulmonary function testing system (Beijing Bestlab High-Tech Co., Ltd.), table-type refrigerated centrifuge (Thermo Fisher), multifunctional microplate reader (TECAN), vertical electrophoresis bath, transfer trough, gel imaging system (Bio-rad, America), doxofylline (Fuhe Huaxing Pharmaceutical Shares Co., Ltd., Heilongjiang), budesonide (Astra-Zeneca, Sweden), HDAC2 (D6S5P), Rabbit monoclonal antibody (Cell signaling technology), Phospho-Akt (Ser473) Rabbit Antibody (Cell signaling technology), Akt (pan11E7), PI3K (p85 alpha), Antibody (Protein tech), Rat IL-8 elisa kit (Shanghai West Biotechnology Co., Ltd.), Rat TNF- α elisa kit (Wuhan USCN Business Co., Ltd.).

Experimental grouping and processing

Simple cigarette exposure was applied in this study to establish COPD model [10]. The rats, normally breed for 7 days without any abnormality, were divided into: cigarette exposure group (CE group, n=30) and control group (n=6). The rats of CE group were placed in homemade glass exposure box, and they were free to move, eat and drink. Cigarettes were lit on the half height of the box with two times per day for 1 h each time (the interval was over 5 h), with 5 days per week and 20 cigarettes each time. After 12 weeks of cigarette exposure, six rats were randomly selected from the CE group to perform pulmonary function testing; and they were sacrificed by abdominal aorta bleeding. Then bronchoalveolar lavage fluid (BALF) was used to lavage the left lung and the lavage fluid was collected to examine the number of inflammatory cells. Afterwards, the lung tissues were stained with HE in order to observe and check whether the model was successfully established.

After successfully establishing the model, 24 COPD rat models were divided into 4 groups for drug intervention with 6 rats in each group and continued cigarette exposure at the same time. The four groups were listed as follows: cigarette exposure group (CE group), cigarette exposure with budesonide group (CE+B group), cigarette exposure with doxofylline group (CE+D group),

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Table 1. Pulmonary function in CE and control group after 12 weeks with cigarette exposure ($\bar{x} \pm sd$)

Group	Control group	CE group	t	P
FVC (ml)	8.72±0.40	8.75±1.75	0.041	0.968
FEV0.3 (ml)	6.60±0.30	5.73±0.89	2.269	0.047
FEV0.3/ FVC	75.66±2.02	66.22±3.69	5.497	<0.001
RI (cm H ₂ O·ml ⁻¹ ·s ⁻¹)	1.31±0.11	2.45±0.76	3.636	0.005
RE (cm H ₂ O·ml ⁻¹ ·s ⁻¹)	1.78±0.16	3.42±0.92	4.302	0.002
Cdyn (ml/cm H ₂ O)	0.20±0.01	0.13±0.02	7.669	<0.001

Note: There was no significant difference in FVC between the two groups. Compared with the control group, FEV0.3, FEV0.3/FVC and Cdyn decreased while RI and RE increased in CE group after 12 weeks with cigarette exposure, and the difference was statistically significant (all $P < 0.05$). All the results indicated the successful establishment of COPD model by cigarette exposure.

Table 2. Inflammatory cells count in BALF in CE and control group after 12 weeks with cigarette exposure ($\bar{x} \pm sd$)

Group	Control group	CE group	t	P
Case	6	6		
Cell count (10 ⁷ /L)	4.1±0.3	7.2±0.6	11.32	<0.001
Cell classification (%)				
Macrophage	85.3±1.5	74.7±4.5	5.587	<0.001
Neutrophil granulocyte	6.7±1.2	17.0±3.6	6.649	<0.001
Leukomonocyte	8.0±1.7	8.3±3.1	0.208	0.840

Note: The total number of cells in BALF in CE group (12 weeks) significantly increased; the proportion of neutrophil granulocyte significantly increased while the proportion of macrophage decreased, which was in line with the changes of COPD.

Table 3. Results of MLI and MAN in CE and control group after 12 weeks with cigarette exposure ($\bar{x} \pm sd$)

Group	Case	MLI (μm)	MAN (μm ²)
Control group	6	41.47±2.36	3.72±0.34
CE group	6	52.39±3.46	2.69±0.25
t		6.387	5.978
P		<0.001	<0.001

Note: MLI value increased while MAN value decreased, which were in line with the changes of COPD related pathology.

and cigarette exposure with budesonide and doxofylline group (CE+B+D group). Nebulized budesonide (2 mg/4 ml) in a closed chamber was proceeded once a day for 14 days; and doxofylline (17.3 mg/kg) was administered via gavage twice a day for 14 days.

Twenty-four hours after the last administration of drugs, the rats of CE group and three drug treated groups were performed pulmonary function testing again. Three rats from each group were sacrificed by abdominal aorta bleeding, and then bronchoalveolar lavage

fluid was used to lavage the left lung; the lavage fluid was collected to examine the number of inflammatory cells; afterwards, the lung tissues were stained with hematoxylin-eosin so as to observe the hormone resistance in COPD rats and the improvement efficacy of doxofylline on hormone resistance. The remaining rats were put to death by carotid artery dislocation and dissected. The fresh lung tissue was used to measure the expression of lung proteins (HDAC2, P-PI3K/PI3K and P-AKT/AKT), the activity of HDAC2 and the expression of IL-8 and TNF-α in serum and the supernatant of BALF, which helped to explore the mechanism.

Methods of pulmonary function testing

The rats were anesthetized by intraperitoneal injection of 3% of pentobarbital sodium (110 mg/kg), after which the trachea was separated and the "T" incision was inverted in the third and fourth tracheal ring. Then the self-made trachea cannula was inserted into the trachea, fastened with surgical sutures and placed into plethysmograph. The trachea was connected to the plethysmograph which inflated with air (3 times the

volume of the tidal volume) to cause deep inspiration; and then release immediately to connect negative pressure (-10 cmH₂O (1 cmH₂O= 0.098 kPa)) to cause deep breath. After that, the volume variation was processed by computer to calculate the forced expiratory volume: forced expiratory volume in 0.3 seconds (FEV0.3), the ration of the forced expiratory volume in 0.3 seconds in the forced vital capacity (FEV0.3/FVC), inspiratory resistance (RI), expiratory resistance (RE), and pulmonary dynamic compliance (Cdyn).

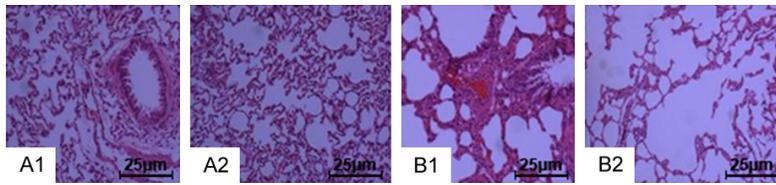


Figure 1. Pathological HE staining of lung tissue in rats treated with cigarette exposure for 12 weeks. A1, A2: Control group, B1, B2: CE group. A1: Bronchial fibers arranged neatly, no inflammatory cells infiltrated and the basement membrane was comparatively thin; A2: Alveolar morphology and size of the lung tissue were normal, and alveolar wall was integrity; B1: Airway epithelium shed, airway adhered, cell obstructed, and bronchial submucosal edema existed; B2: The damage of the lung tissue structure increased, alveolar expanded, alveolar septum thickened, partial alveolar wall destructed, the phenomenon of adjacent alveolar fusion to form bullae of lung appeared, and small vascular wall thickened. All the results were in agreement with the changes of COPD related pathology.

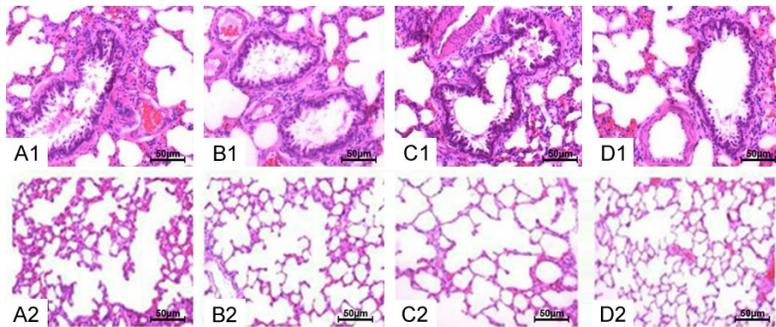


Figure 2. Pulmonary pathology of COPD rat models after drug intervention. A1-D1: airway pathological morphology observation; A2-D2: alveolar pathological morphology observation. A1, A2: CE group; B1, B2: CE+D group; C1, C2: CE+B group; D1, D2: CE+B+D group. Compared with the CE group, the falloff of airway epithelium decreased, airway adhesion and cell blockage reduced, the damage of the lung tissue reduced, the phenomenon of adjacent alveolar fusion to form bullae of lung decreased and COPD related pathological changes were alleviated in the CE+D group, and the improvement was more obvious in the CE+B+D group. However, in CE+B group, the glucocorticoid resistance phenomenon appeared, bronchial inflammation infiltration enhanced, the phenomenon of mucinous gland hyperplasia and many mucosal wrinkles showed no significant improvement, meanwhile, the phenomenon of adjacent alveolar fusion to form bullae of lung was obvious, and the symptoms of the lung tissue presented less improvement.

BALF collection and inflammatory cell counting in BALF

The rats were sacrificed by abdominal aorta bleeding before which blood from the abdominal aorta was collected in EP tube and stored at -20°C. The trachea was intubated to the left main bronchus with the rat gavage needle; and the left lung was perfused with serum-free RPMI 1640 medium for 5 ml. Meanwhile, the lungs were massaged for 30 s which repeated for 8 times with a recovery rate of 90%. The

recovered BALF was placed in the conical centrifuge tube and centrifuged at the speed of 1,000 r/min for 10 min and then the supernatant was withdrawn. After that, cells were resuspended in 1 ml RPMI 1640 medium and fully blown and mixed. The number of cells in 10 µl cell suspensions was counted with a blood counting chamber; in addition, 100 µl cell suspension was collected and smeared by cytospin and dried naturally; after Wright-Giemsa staining, the inflammatory cells were classified and counted. Specifically, 100 cells were counted under the oil microscope and the macrophages, neutrophile granulocyte and leukomonocytes were counted according to the cell morphological characteristics with the ratio calculated (percentage) [11].

Pathological observation of lung tissue

The right main bronchus was ligated and the anterior lobe of the right lung was placed into the EP tube. The thick tissue blocks about 3 mm, taken from the largest diameter of the middle lobe of the right lung, was immersed in 4% of paraformaldehyde for 48 hours and then was embedded in paraffin. Three slices were cut along different directions and then stained with and observed with light microscope.

Morphological quantitative analyses of lung tissue was performed by Imge-pro Plus. The specific steps of mean linear intercept (MLI) measurements were as follows: three HE staining sections were taken from each rat and put under the low magnification (100X) to randomly take 5 visual views (to avoid large vessels and

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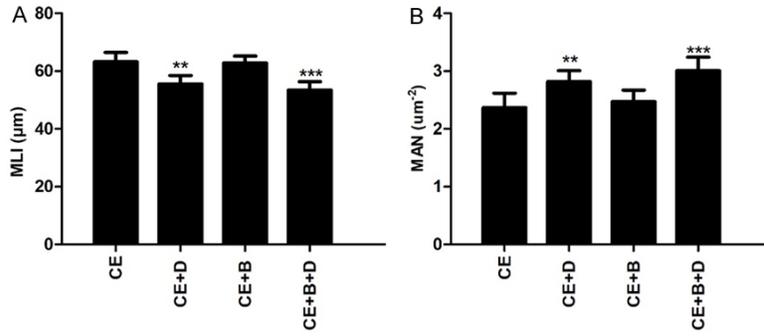


Figure 3. Quantitative results of lung tissue morphology after drug intervention. A: Mean linear intercept, MLI; B: Mean alveolar numbers, MAN; compared with CE group, ** $P < 0.01$, *** $P < 0.001$.

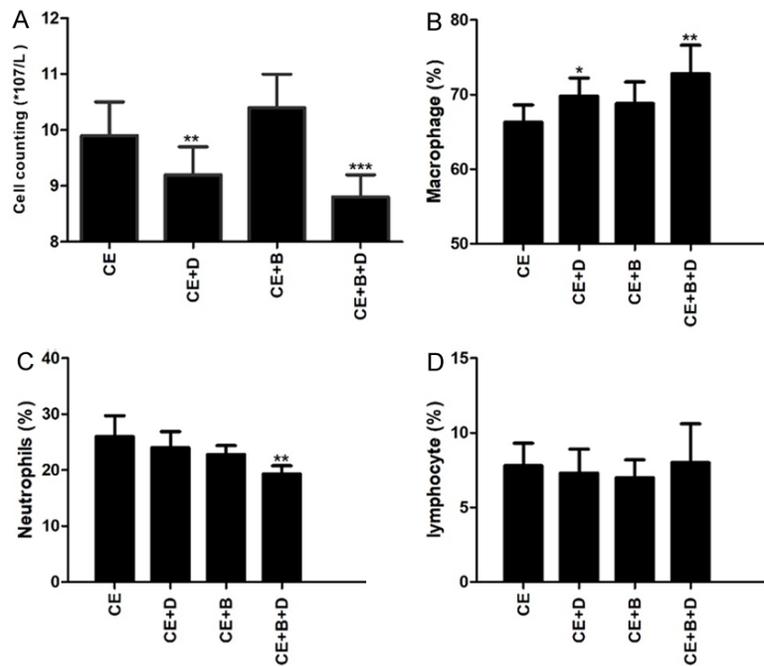


Figure 4. Inflammatory cells count in BALF after drug intervention. A: Cell counting; B: Macrophage; C: Neutrophil granulocyte; D: Lymphocyte; compared with CE group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

bronchi); and at the center each visual field, the cross lines were drawn to count the number of alveolar septum (NS) intersecting the cross lines, and measure the total length of the cross (L) at the same time; mean alveolar diameter was calculated according to the formula: $\text{MLI} = \text{L}/\text{NS}$; mean alveolar numbers (MAN) was calculated to reflect the alveolar density on the basis of the formula: $\text{MAN} = \frac{\text{the number of alveolar counted for each field}}{\text{the area of this view}}$. Ten visual fields were measured in each sample and the average value was obtained [12].

of morphology, the count of inflammatory cells in alveolar lavage fluid were proceeded on the rats treated with CE (Tables 1-3, and Figure 1). The results above suggested that the COPD model was established successfully and the subsequent experiment results were reliable.

Results of drug intervention in COPD rats model

General information of rats: Rats in CE group were marasmic and had less activity, poor spirit and intermittent cough with occasional pant-

Detection of the level of proteins and inflammatory factors of the lung tissue

The expression of HDAC2, P-PI3K/PI3K, P-AKT/AKT in the lung tissue was detected by western blot; the level of IL-8 and TNF- α in the serum and BALF was measured by enzyme linked immunosorbent assay; the activity of HDAC2 in the lung tissue was detected by using HDAC2 activity assay kit.

Statistical analysis

Data were analyzed by the SPSS.19.0 statistical software and presented as mean \pm standard deviation; one-way analysis of variance was applied for comparison among groups and the SNK-q test was used for the comparison of the two groups. Pearson correlation analysis was used to analyze the relationship between the different parameters. $P < 0.05$ was considered that differences were statistically significant.

Results

Detection of COPD model result

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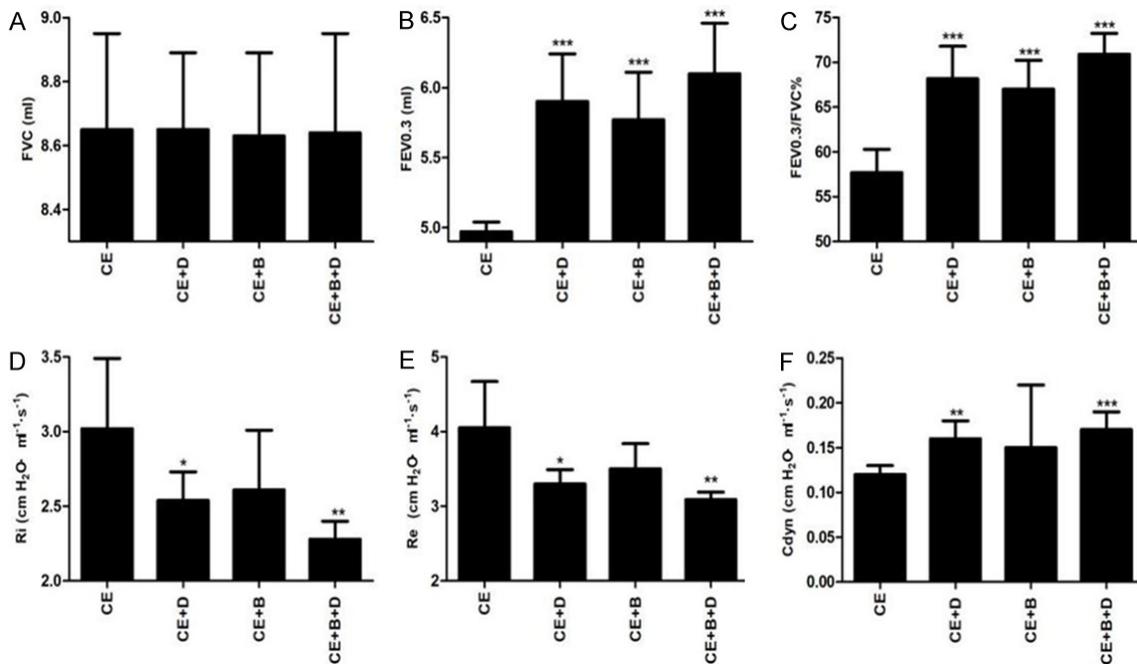


Figure 5. Pulmonary function results after drug intervention. A: FEV; B: FEV0.3; C: FEV0.3/FVC; D: RI; E: RE; F: Cdyn; compared with CE group, *P<0.05, **P<0.01, ***P<0.001.

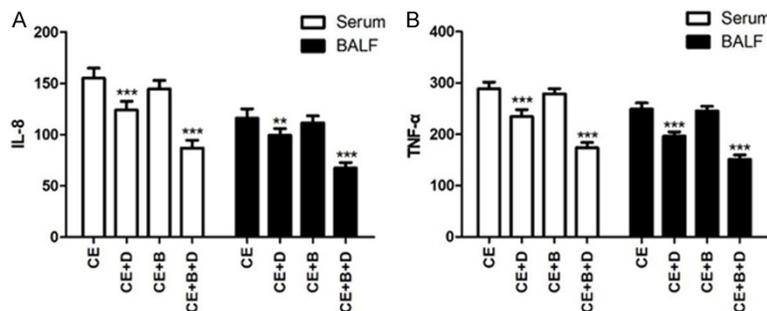


Figure 6. IL-8 and TNF- α expression in serum and BALF after drug intervention ($\bar{x} \pm s.d.$). Compared with CE group, *P<0.01, ***P<0.001.

ing. Besides, their hair dried, yellowed and fell off, and their lips and finger claws had cyanosis. Compared with CE group, rats in CE+D group and CE+B+D group had better spirit, more activities and the cyanosis in lips and finger claws was less obvious; rats in CE+B group had no significant improvement.

Results in pathological changes and morphological quantitative analysis after drug intervention: Compared with the CE group, the level of inflammatory cell infiltration was significantly lower in the CE+D group; the improvement was not obvious in the CE+B group, and the level of inflammatory cell infiltration in alveolus and

bronchus was comparatively higher; the level of the inflammatory cell infiltration descended in the CE+B+D group and the alveolar injury was not serious (Figure 2).

The results of MLI and MAN are shown in Figure 3. Compared with the CE group, MLI decreased in the CE+D group and CE+B+D group (all P<0.05), and the decrease was more obvious in the CE+B+D

group. The decrease in the CE+B group was slight but not significant (P>0.05). Compared with the CE group, MAN had different degrees of increase in the three drug intervention groups, and the MAN value increased significantly in the CE+D group and CE+B+D group (P<0.05).

Results in inflammatory cells count in BALF after drug intervention

The total number of cell in CE+B+D group (P<0.001) and CE+D group (P=0.004) in BALF were significantly lower than that in CE group. Specifically, compared with CE group, the number of macrophage in CE+B+D group (P=0.005)

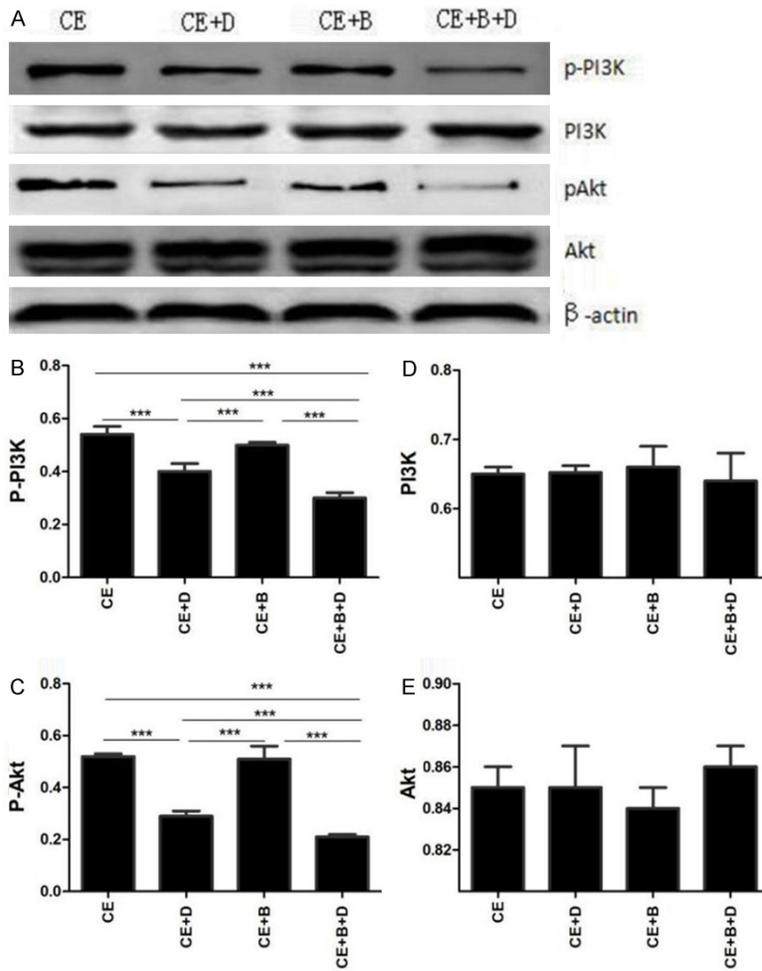


Figure 7. P-PI3K/PI3K and P-Akt/Akt protein expression results in lung tissue after drug intervention. A: Western blot figure of P-PI3K/PI3K, P-Akt/Akt protein; B-E: Protein quantitative figure; β -actin as the internal reference; *** $P < 0.001$.

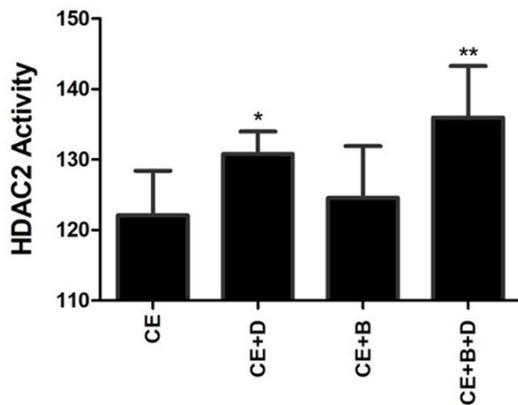


Figure 8. HDAC2 protein activity level in lung tissue after drug intervention. Compared with CE group, * $P < 0.05$, ** $P < 0.01$.

and CE+D group ($P=0.03$) was obviously increased; the number of neutrophil granulocytes was decreased significantly in CE+B+D group ($P=0.002$) and slightly decreased in CE+D group and CE+B group (both $P > 0.05$); there were little significant differences in the change of white blood cells count in each group (all $P > 0.05$) See **Figure 4**.

Results of pulmonary function after drug intervention: There were no significant changes in forced vital capacity (FVC) in each group. Compared with CE group, the value of FEV0.3 was increased at different degrees in CE+D group (5.90 ± 0.34 ml), CE+B group (5.77 ± 0.34 ml) and CE+B+D group (6.10 ± 0.36 ml), (all $P < 0.001$); the value of FEV0.3/FVC also had different degrees of increase in CE+D group, CE+B group and CE+B+D group (all $P < 0.001$); the RI and RE values were decreased significantly in CE+D group ($P=0.043$, $P=0.004$) and CE+B+D group ($P=0.018$, $P=0.004$) and slightly decreased in CE+B group ($P=0.134$, $P=0.086$);

the Cdyn value was raised in CE+D group ($P=0.0014$) and CE+B+D group ($P < 0.001$) and slightly raised in CE+B group without statistical significance ($P=0.323$) See **Figure 5**.

IL-8 and TNF- α expression in serum and BALF after drug intervention

Compared with the CE group, IL-8 and TNF- α expression in serum and BALF in CE+B+D group decreased significantly (all $P < 0.001$); the expression of the two indicators in serum (both $P < 0.001$) and BALF (both $P < 0.01$) in CE+D group also decreased significantly; but there were no significant differences in the change of IL-8 and TNF- α expression in serum ($P=0.072$, $P=0.168$) and BALF

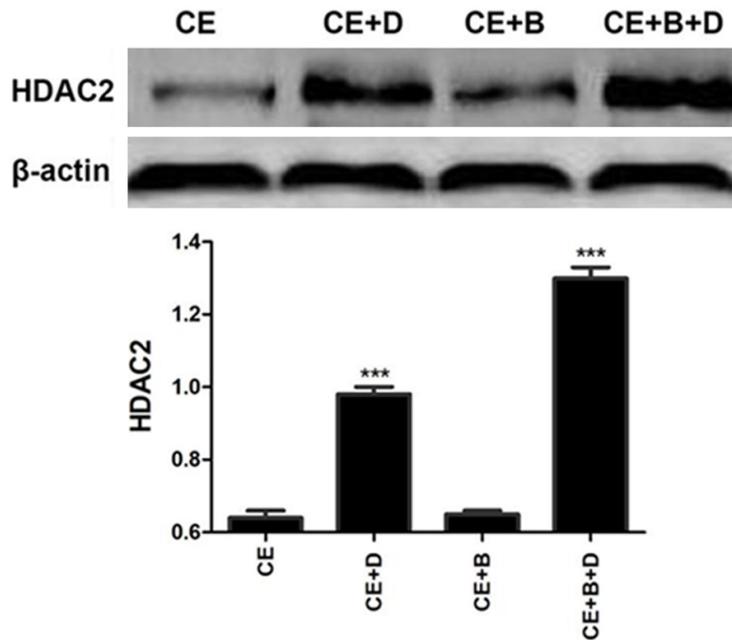


Figure 9. Western blot figure of HDAC2 protein in lung tissue after drug intervention. Compared with CE group, *** $P < 0.001$.

Table 4. Correlation analysis of HDAC2 protein activity and related indicators

	Serum IL-8	BALF IL-8	Serum TNF- α	BALF TNF- α	FEV0.3	MLI
r	-0.699	-0.762	-0.742	-0.721	0.727	-0.823
P	0.002	0.001	0.001	0.002	0.001	0

($P = 0.339$, $P = 0.5643$) in CE+B group. See **Figure 6**.

P-PI3K/PI3K and P-Akt/Akt protein expression in lung tissue after drug intervention

Lung tissue proteins were extracted and the protein expression condition of PPI3K/PI3K and P-Akt/Akt was detected by western blot. There were no significant differences in PI3K, and Akt gray values from lung tissue of rats among groups (all $P > 0.05$). There were no significant differences in gray values of P-PI3K between CE+B group and CE group ($P = 1.000$), indicating that the expression of P-PI3K protein in the two groups did not change significantly; compared with CE group, the expression of P-PI3K protein in CE+B+D group and CE+D group fell significantly (both $P < 0.001$); meanwhile, there was significant difference in P-PI3K protein expression between CE+B+D group and CE+D group. As for the P-Akt, there were also no significant differences of gray values

between CE+B group and CE group ($P = 0.064$), indicating that the expression of P-Akt protein in the two groups did not change significantly; compared with CE group, the expression of P-Akt protein in CE+D group and CE+B+D group fell significantly (both $P < 0.001$). See **Figure 7**.

Protein activity and expression of HDAC2 in lung tissue after drug intervention

HDAC2 protein activity measured in each group is shown in **Figure 8**. Compared with CE group, HDAC2 protein activity in lung tissue of rats in CE+D group and CE+B+D group all increased significantly ($P = 0.021$, $P = 0.006$) while no obvious change was presented in CE+B group ($P = 0.545$). Proteins in lung tissues were extracted respectively from the four experimental groups for western blot examination. The results are shown in **Figure 9**. Compared with CE group, HDAC2 protein expression of rats in CE+D group and CE+B+D group both increased

significantly (both $P < 0.001$); and there were no significant differences in HDAC2 protein expression between CE+B group and CE group ($P = 0.299$). See **Figure 9**.

Correlation analysis of HDAC2 protein activity and related indicators in lung tissue after drug intervention

As shown in **Table 4** and **Figure 10**, HDAC2 protein activity in lung tissue was negatively correlated with level of IL-8 and TNF- α in serum and supernatant of BALF; HDAC2 protein activity in lung tissue was positively correlated with lung function indicator of FEV0.3 and negatively correlated with MLI.

Discussion

The animal models established by CE can replicate the major pathogenesis of human COPD and are widely used in the study of pathogenesis and treatment of COPD [13-15]. This

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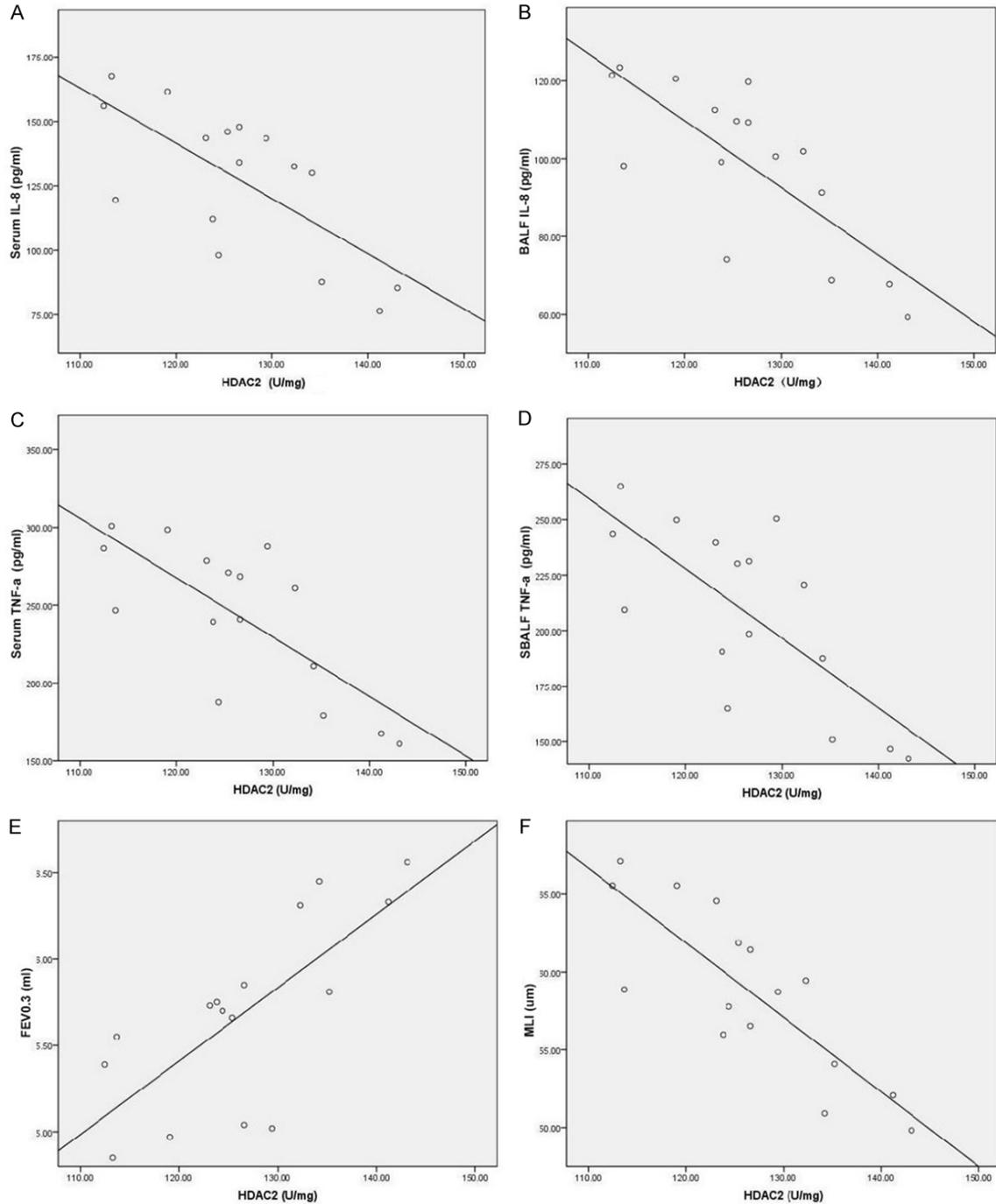


Figure 10. Scatter diagram of correlation analysis of HDAC2 protein activity and related indicators.

research adopted simple CE for 12 weeks to establish the COPD rat model. After 12 weeks' CE, the pulmonary tissue pathology, pulmonary function test and inflammatory cell count of BALF were done to evaluate whether the establishment of COPD rat model was successful or not. The results showed that the lung pathology

of rats exposed to cigarette for 12 weeks was accord with the lung pathological changes of COPD, the lung function decreased, the total number of inflammatory cells in BALF increased, and the neutrophils were predominant. Therefore, the COPD rat model was successfully established in this study and could be

used for the study of drug therapy in the later stage.

Although GC was still a commonly used clinical drug for COPD at present, whether inhaled alone or taken orally, the effect of GC treatment was not significant [16-18]. This study found that compared with CE group, the IL-8 and TNF- α levels in serum and BALF of rats in CE+B group were not significantly different, MLI, MAN and other lung tissue damage quantitative indicators also showed no significant difference, indicating that the airway inflammation and emphysema in rats were not improved, and hormone resistance existed. The hormone resistance in COPD patients might be related to the decrease of HDAC2 activity and expression [19, 20]. The results of this study suggested that although the activity and expression of HDAC2 increased slightly after budesonide treatment, there was no significant difference. It means that further research on related mechanisms is needed.

Previous studies have showed that the combined application of small doses of theophylline and GC can effectively increase the sensitivity of COPD hormone, which might be related to the effect of theophylline on the recovery of the expression and activity of HDAC2 [21, 22]. But whether doxofylline could increase the expression of HDAC2 like theophylline was rarely reported. The results of this research showed that compared with the CE group, the expression and activity of lung tissue protein HDAC2 in CE+D group was increased, suggesting that doxofylline could increase the expression and activity of the HDAC2 like theophylline, thereby reduced the IL-8, TNF- α levels in serum and bronchoalveolar lavage fluid. Compared with CE+B group, the expression and activity of HDAC2 in CE+B+D group was increased significantly, the levels of IL-8 and TNF- α in serum and BALF was decreased obviously, and the activity of HDAC2 in lung tissue was negatively correlated with the secretion levels of IL-8 and TNF- α , MLI, and positively correlated with the lung function index FEV_{0.3}. It illustrated that doxofylline could reduce the expression of inflammatory factors and reverse the hormone resistance of COPD rat model by increasing the expression and activity of HDAC2 in lung tissue of COPD rat model.

At present, it is thought that the most important mechanism of oxidative stress in leading to the

changes in HDAC2 expression and activity is the activation of the PI3K/Akt pathway [23]. A study showed that PI3K δ and P-Akt increased in COPD rats, while HDAC2 decreased significantly [24]. This study found that compared with CE group, P-PI3K and P-Akt protein expression in lung tissues decreased significantly in CE+B group and CE+D group, and the expression and activity of HDAC2 also increased significantly. Compared with the CE group, the expression of two proteins group and the expression and activity of HDAC2 in the CE+B were not significantly changed. It showed that doxofylline might improve the hormone resistance of COPD patients by reducing the expression of P-Akt and P-PI3K and then influencing the activity of HDAC2. But the results of this study only suggested that doxofylline could decrease the expression of P-PI3K and P-Akt protein in lung tissue of COPD rat model. There was no evidence to confirm that doxofylline had a direct inhibitory effect on PI3K α , β , γ , δ and other hypotypes. Therefore, the exact pathway of doxofylline and hormone's synergistic reversal of HDAC2 activity is the focus of the study in the future.

In summary, the combined use of hormone and doxofylline can improve airway inflammation and emphysema degree of COPD rat model. This might be associated with the increased expression and activity of HDAC2 which is caused by the down-regulation of P-PI3K and P-Akt protein expression in lung tissue of rats. At last, a conclusion can be drawn that doxofylline can improve the hormone resistance of COPD model rats by reversing the activity of HDAC2.

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Disclosure of conflict of interest

None.

Address correspondence to: Nengluan Xu, The Shengli Clinical Medical College of Fujian Medical University, Fujian Provincial Hospital, and Fujian Medical University, Fuzhou 350001, Fujian Province, China; Department of Respiratory and Critical Care Medicine, Fujian Provincial Hospital, No.134 East Street, Fuzhou, 350001, Fujian Province, China; The

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Laboratory of Four Respiratory Diseases, Fujian Provincial Hospital, No.134 East Street, Fuzhou 350001, Fujian Province, China. Tel: +86-138050-88136; E-mail: XNL68686@163.com

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