Effect of Myrtol standardized on mucus hypersecretion and clearance of *Pseudomonas aeruginosa* in a rat model of chronic obstructive pulmonary disease

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Abstract

This study aimed to investigate the effect of Myrtol standardized (GeloMyrtol® forte) in the treatment of chronic obstructive pulmonary disease (COPD) in an animal model. A total of 93 experimental rats were randomly divided into 6 groups: control (n = 6), exposure to cigarette smoke (CS, n = 6), CS plus Myrtol standardized treatment (CS + M, n = 6), *Pseudomonas aeruginosa* (PA) infection, PA + M (n = 25), CS + PA (n = 25), and CS + PA + M (n = 25). For all 62 CS rats, they were exposed to cigarette smoke for a period of 12 weeks. During this time period the 31 CS + M rats (CS + M; CS + PA + M) received 300 mg/kg/day Myrtol standardized intragastrically always 30 min prior to smoke exposure. For CS + PA and CS + PA + M rats, intratracheal PA inoculation was performed after the 12 weeks of smoke exposure. All intratracheal PA inoculations were followed by a post-infection examination at 6, 12, 24, 48 and 72 h in each 5 rats. All study animals were euthanized and their lungs were excised; the left lung was homogenized for determination of bacterial load and measurements of TNF-α and IL-6, the right lungs were preserved for histo- and immunohistochemical examinations (e.g., MUC5AC). The lungs from CS rats were pathologically similar to those of COPD patients with the characteristics of goblet cell metaplasia and MUC5AC hypersecretion. CS animals had a significantly greater number of MUC5AC positive cells in the bronchial epithelial cells, and significantly increased expression levels of TNF-α and IL-6 after PA infection. However, the administration of Myrtol standardized significantly (p < 0.002) attenuated MUC5AC hypersecretion, measured as integrity optical density (IOD), in CS+M rats (45.98 ± 6.25) as compared to CS alone (65.55 ± 11.18) rats. The same applies at different time points between CS + PA rats (65.15 ± 11.94, 75.88 ± 7.42, 81.2 ± 6.49, 75.14 ± 6.85 and 67.32 ± 10.61, respectively) and CS + PA + M rats (47.98 ± 4.78, 54.22 ± 6.59, 56.4 ± 6.12, 59.98 ± 4.96 and 48.43 ± 7.29, respectively). Similar effects were found in the production of IL-6 and TNF-α in the CS + PA + M lungs. Similarly the bacterial load of 10,980 ± 4,253 CFU in CS + PA + M was significantly lower compared to 42,400 ± 3,296 CFU in CS + PA lungs after 72 h PA infection. In conclusion, this experimental study demonstrates a significant therapeutic effect of Myrtol standardized in treating common pathological conditions, such as airway mucus hypersecretion and defect of mucociliary functions in COPD.

Key words

- Bacterial clearance
- Chronic obstructive pulmonary disease
- Mucus production
- Myrtol standardized
- *Pseudomonas aeruginosa* infection

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1. Introduction

Airway mucus hypersecretion is a characteristic feature of chronic inflammatory diseases such as chronic obstructive pulmonary disease (COPD), asthma and bronchiectasis. Mucus overproduction in the airways greatly contributes to decreased mucociliary transport [1]. Mucociliary dysfunction can lead to an accumulation of mucus and bacterial colonization in the lower airway, which can result in the likelihood of recurrent respiratory infections and the exacerbation of COPD [2]. Repeated exacerbations may potentially lead to an accelerated rate of decline in pulmonary function and an increase in airway inflammation and mortality [3]. Current pharmacotherapeutic options for acute exacerbation of COPD are anti-inflammatory drugs (like glucocorticosteroids), bronchodilators (like β-2-sympathomimetics, theophylline, anticholinergics) and mucolytics which can influence mucus viscoelasticity [4, 5]. New therapies are urgently needed to treat mucus hypersecretion.

Myrtol standardized is an essential oil that contains primarily cineole, limonene and α-pinene. Several studies have demonstrated that Myrtol standardized can prevent impairment of lung function of COPD patients and decrease the frequency and severity of acute exacerbations [6]. Moreover, clinical trials have shown that Myrtol standardized can increase mucociliary clearance [7]. Myrtol standardized has shown secretolytic, mucolytic and secretomotor effects [8]. It is unknown, however, whether Myrtol standardized affects mucus hypersecretion and bacterial clearance.

We postulate that Myrtol standardized may promote the clearance of respiratory bacteria by reducing airway mucus, and thus may relieve airway obstruction and reduce exacerbations of COPD. It is known that Myrtol standardized has anti-inflammatory properties [9] by reducing the leucotriene concentration (LTC4/D4/E4) and inhibition of 5-lipooxygenase [10]. In this study, we used a rat model of COPD for investigating the impact of Myrtol standardized on bacterial clearance, proinflammatory cytokines and airway mucus hypersecretion.

2. Materials and methods

2.1 Study animals

Ninety-three male Wistar rats weighing 180–220 g were purchased from the Experimental Animal Centre of China Medical University. Animals were treated according to the principles of laboratory animal care, as well as the current version of Chinese Law on the Protection of Animals. Rats were randomly divided into 6 groups: control (n = 6), exposure to cigarette smoke (CS, n = 6), CS plus Myrtol standardized treatment (CS + M, n = 6), Pseudomonas aeruginosa (PA) infection (PA, n = 25), CS + PA (n = 25), and CS + PA + M (n = 25).

2.2 Cigarette smoke exposure protocol

All 62 CS rats (CS; CS + M; CS + PA; CS + PA + M) were exposed to cigarette smoke for a period of 12 weeks [11]. Briefly, the rats were exposed to side stream cigarette smoke (“Great front door” cigarettes with filters, Shanghai Tobacco, Inc., China. Tar: 15 mg/cigarette and nicotine: 1.1 mg/cigarette) in a whole body smoke exposure chamber. Rats were exposed to cigarette smoke for 30 min twice a day. Twenty-eight cigarettes were used per day. The same brand of locally available cigarette was used in every experiment. During this time period, Myrtol standardized (GeloMyrtol® forte, batch number: 126058, Pohl Boskamp, Hohenlockstedt, Germany) was administered intragastrically at a dosage of 300 mg/kg/day 30 min prior to smoke exposure in the CS + M and CS + M + PA rats. All groups without Myrtol standardized (control, CS, PA, CS + PA) received a corresponding volume of normal saline.

2.3 P. aeruginosa infection

P. aeruginosa (ATCC 27853) was kindly donated by the Microbiology Lab of China Medical University. Bacteria were harvested from solid overnight plate cultures and suspended in phosphate buffered saline (pH 7.4). Concentrations of bacteria were estimated by measuring the optical density [12] and were confirmed by plate count. Approximately 10^8 CFU were used to infect all PA rats (PA; CS + PA; CS + PA + M). After 12 weeks of exposure to smoke, the PA, CS + PA and CS + PA + M rats were anesthetized with 10% chloral hydrate (3.5 ml/kg) and infected intratracheally. At the time point of 6 h, 12 h, 24 h, 48 h and 72 h post-infection, rats were euthanized and their lungs were excised; the left lung was homogenized for determination of bacterial load and measurements of TNF-α and IL-6, the right lungs were preserved for histo- and immunohistochemical examinations (e.g. MUC5AC; see below). The bacterial load in the inocula was determined by colony count.

2.4 Histopathology

For histologic evaluation, lungs were fixed in 4% polyformaldehyde for 24 h and embedded in paraffin. 5 μm thick sections were stained with hematoxylin and eosin. The pathomorphology was examined using a pathology image analysis system (BX51 Olympus Japan) spot insight color (Diagnostic instruments Inc USA), Metamorph software (Universal Imaging Corporation, USA). Five visual fields in one microtome section were selected along the maximal trans and lengthways diameter using a magnification of 10 × object lens. The mean linear intercepts (MLI) and mean alveoli number (MAN) were calculated to evaluate the degree of pulmonary emphysema [13]. The MLI represents the average size of alveoli respectively the enlargement of air spaces. Paraffin sections were stained with alcin blue-periodic acid-Schiff (AB-PAS) to assess goblet cell metaplasia. A goblet cell count was conducted by assessing 500 bronchial epithelial cells on an image analyzer (Universal Imaging Corporation, USA) [14].

2.5 Immunohistochemistry

Immunohistochemical detection of MUC5AC was performed on paraffin-embedded tissue sections using the avidin-biotin-peroxidase technique. Following paraffin removal and dehydration of sections, antigens were retrieved from each slide by microwave them for 15 min in 0.01 mol/L citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide for 10 min. Samples were blocked by incubation with 5% bovine serum albumin (BSA). Sections were subsequently incubated with polyclonal anti-MUC5AC (1:100 dilution, SC-71620, Santa Cruz) antibody over night at 4°C. Control sections were incubated in PBS. Antibodies were detected using the SABC kit (SA1021, Boster) according to the manufacturer’s protocol. Antibodies were visualized.
using the diaminobenzidine kit (AR1022, Boster) and images were analyzed using the Metamorph software (Universal Imaging Corporation, USA).

2.6 Measurements of tissue TNF-α and IL-6

TNF-α and IL-6 concentrations in lung homogenate specimens were measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific ELISA kits according to the manufacturer’s instructions (Genetimes Technology Inc., China).

2.7 SYBR Green Real-Time RT-PCR

Total RNA in lung tissues was isolated using Trizol reagents (D9108A, TaKaRa). The cDNA was synthesized by reverse transcription polymerase chain reaction (RT-PCR) according to the manufacturer’s instructions (DRR081A, TaKaRa). Reverse transcription condition: 37 °C for 15 min, 85 °C for 4 s. Primers for MUC5AC were: forward 5’-CTG CCA ACC CTT ACC G-3’, reverse 5’-GCA CAG GCG TCA TTC A-3’; and for GAPDH: forward 5’-GCA CAG GCG TCA TTC A-3’, reverse 5’-CAT TTG ATG TTA GCG GGA T-3’. Real-time PCR reaction was performed using the ABI PRISM 7500 Real Time PCR System (Applied Biosystems, USA): pre-denaturation for 30 s at 95 °C, PCR reaction 5 s at 95 °C, for 34 s at 60 °C, for a total of 40 cycles. The threshold cycle (Ct) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. Relative expression of MUC5AC mRNA was calculated by the 2−ΔΔCt method [15].

2.8 Statistical analysis

Data analysis was performed using the statistical software SPSS13.0. All data were expressed as mean ± SD. Differences between groups were analyzed using the one-way ANOVA and Fisher’s post-hoc test. A P value of less than 0.05 was considered a statistically significant difference.

3. Results

3.1 Status of the rats

The health status of the experimental animals was observed based on appearance, activity, breathing pattern and appetite. At the end of the study the rats in the control group had fair, clear glossy fur, normal behaviour and appetite. In contrast, the rats in the cigarette smoke groups (CS, CS + M) showed withered and yellow fur, lassitude and decreased activity; the animals in the P. aeruginosa infection groups (PA, CS + PA, CS + PA + M) had a frequent and short breath, poor appetite, listlessness, rolled up, upright fur and physical inactivity. The rats in the Myrtol standardized groups showed good tolerance to the drug, without any side effects.

3.2 Histopathological examination of lung tissues

Histopathological changes in lung tissues of smoke-treated rats showed significant pulmonary damage compared with those in the control group (Fig. 1A). The alveolar wall became thin and broken and was accompanied by an enlargement of the alveolar space (Fig. 1C, 1E). Moreover, mean linear intercepts (MLI) were increased in all smoke-treated groups, but mean alveoli numbers (MAN) were significantly lower than that of the control group (Table 1). Taken together, chronic smoke exposure induced lesions similar to COPD/empysema. We observed that a large number of neutrophils had infiltrated the pulmonary interstitium and alveolar space 72 h after P. aeruginosa infection and that lung consolidation had occurred (Fig. 1B, 1F). In particular, it seemed that larger areas of consolidation occurred in the CS + PA group (Fig. 1D).

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Fig. 1: Light photomicrograph of paraffin-embedded sections of lung tissues. Lung tissues were collected before and 72 h after inoculation with P. aeruginosa. Lung sections were stained with hematoxylin and eosin, with a magnification ×100. A, control group. B, PA group (rats were inoculated with 10⁸ CFU P. aeruginosa). C, CS group (rats were exposed to cigarette smoke for 12 weeks). D, CS + PA group (rats were exposed to cigarette smoke and inoculated with P. aeruginosa). E, CS + M group (rats were exposed to cigarette smoke and given Myrtol standardized). F, CS + PA + M group (rats were exposed to cigarette smoke and inoculated with P. aeruginosa and given Myrtol standardized).
3.3 Goblet cell metaplasia
To assess the effect of Myrtol standardized on airway mucus hypersecretion induced by cigarette exposure, we stained paraffin sections with alcian blue-periodic acid-Schiff (AB-PAS). Staining of airway tissue sections allows mucins to be easily identified in goblet cells. In control rats, AB-PAS positive cells (0.034 \pm 0.018) were rarely observed in the airway epithelium. However, we observed that the number of AB-PAS positive cells (0.252 \pm 0.089) was significantly increased after prolonged exposure to smoke. This increase in AB-PAS positive cells after smoke exposure could be significantly attenuated (p < 0.05) by the administration of Myrtol standardized (0.148 \pm 0.056) (Fig. 2).

3.4 MUC5AC expression in the lung
The mucoprotein MUC5AC is predominantly observed in the mucosa epithelium and submucosal gland of the trachea and bronchi. We observed that the level of MUC5AC in the bronchial epithelial cells of smoke-exposed (CS) rats (65.55 \pm 11.18) was significantly (p < 0.05) higher than that in the control group (11.22 \pm 2.95). Likewise, compared with control rats (PA rats), smoke-exposed animals had significantly greater levels of MUC5AC in the bronchial epithelial cells after P. aeruginosa inoculation. In all cases the increased levels of MUC5AC in bronchial epithelial cells upon smoke exposure (CS) and smoke exposure and P. aeruginosa infection (CS + PA) were reduced in rats that were treated with Myrtol standardized (CS + M; CS + PA + M) (Fig. 3). Moreover, significantly increased expression levels of MUC5AC mRNA were detected in smoke-treated (CS) rats before and 12 h and 72 h after P. aeruginosa inoculation (CS + PA), and this also was significantly attenuated by the administration of Myrtol standardized (CS + PA + M) (Fig. 4).

3.5 Bacterial clearance
Similar amounts of P. aeruginosa were cultured from the lungs of each group 6 h after bacterial inoculation. In the control group (PA group), the bacterial counts declined rapidly over time, and bacteria in lungs were almost completely eliminated after 72 h. Smoke-exposed (CS + PA) animals had a significantly greater bacterial load in the lungs at 12, 24, 48 and 72 h after inoculation. Exposure to Myrtol standardized significantly enhanced bacterial clearance at 72 h post-infection (Fig. 5).

![Fig. 2: Histopathological examination of lung tissue sections stained with AB-PAS. A, AB-PAS positive cells were detected in lung tissue sections (with a magnification of \( \times 200 \)). (A1) control group, (A2) CS group, (A3) CS + M group. B, The numbers of AB-PAS positive cells were assessed by image analysis (mean \( \pm SD \); n = 5). *p < 0.05 CS group vs. control group. **p < 0.05 CS + M group vs. CS group (CS: cigarette smoke; M: Myrtol standardized).](image-url)
Fig. 3: Immunohistochemical examination of MUC5AC expression.
A, Representative photomicrographs showing immunohistochemical staining for MUC5AC in (A1) control group, (A2) CS group, (A3) CS + group, (A4) PA group, (A5) CS + PA group and (A6) CS + PA + M group. Lung tissues were collected before and 72 h after inoculation with P. aeruginosa (with a magnification of ×400).
B, Integrity optical density (IOD) of MUC5AC staining was assessed by image analysis before and 6, 12, 24, 48, 72 h after inoculation with P. aeruginosa (mean ± SD; n = 5/time point). *P < 0.05 CS resp. CS + PA group vs. control resp. PA group at each time point. **P < 0.05 CS + M resp. CS + PA + M group vs. CS resp. CS + PA group at each time point (CS: cigarette smoke; PA: P. aeruginosa; M: Myrtol standardized).

3.6 TNF-α and IL-6 expression in lung homogenates
To better characterize the increase in inflammation observed in rats exposed to smoke and P. aeruginosa infection, and to examine the effects of Myrtol standardized on airway inflammation, we measured TNF-α and IL-6 levels in lung homogenates. Compared to the control group (TNF-α: 159.08 ± 46.65 pg/ml; IL-6: 411.5 ± 26.6 pg/ml), smoke-exposed (CS) animals had significantly (p < 0.05) higher levels of the pro-inflammatory cytokines TNF-α and IL-6 before (CS) and after P. aeruginosa (CS + PA) inoculation. Treatment with Myrtol standardized (CS + PA + M) significantly inhibited the pro-inflammatory cytokine expression that was induced by smoke exposure and P. aeruginosa inoculation (Fig. 6).

4. Discussion
Mucociliary clearance (MCC) is a very complex and vulnerable defensive mechanism of the respiratory system [16]. The mucociliary transport system is primarily comprised of cilia and mucus. Mucus and mucins are over-produced in the airways of patients with COPD, which greatly contributes to decreased mucociliary transport [17]. Decreased mucociliary transport leads to an accumulation of mucus within the airways. This leads to an increase in bacterial colonization in the lower airway and the likelihood of recurrent respiratory infections. Infection is the predominant cause of exacerbations of COPD. Recent clinical trials have demonstrated that Myrtol standardized can prevent impairment of lung func-
tion and airway constriction in COPD patients and decrease the frequency and severity of exacerbations [6]. Previous studies showed that Myrtol standardized has beneficial effects on MCC [8]. However, little is known regarding the effects of Myrtol standardized on mucus hypersecretion and bacterial clearance. Our findings suggest that Myrtol standardized promote the clearance of respiratory bacteria and reduce mucus hypersecretion.

Cigarette smoking is recognized as the most important risk factor for development of COPD. Exposure to cigarette smoke may damage ciliary epithelium and promote mucus hypersecretion, which lead to mucociliary dysfunction [18]. MUC5AC is the predominant airway mucin secreted by goblet cells. The increase of MUC5AC gene expression upon exposure to smoke may indicate goblet cell hyperplasia and mucus hypersecretion [19]. In this study, we investigated the effect of Myrtol standardized on the proliferation of goblet cells and the secretion of mucoprotein MUC5AC in a rat model of COPD. We show that rats exposed to cigarette smoke over a 12 week period develop a COPD-like condition, as the histopathologic and morphological alterations of rat lungs are characteristic of human bronchitis and emphysema [20]. We also observed increased levels of mucins in the airway epithelia of rats exposed to cigarette smoke, suggesting goblet cell metaplasia. Using RT-PCR and immunohistochemistry assays, we demonstrated that smoking increases both the mucin MUC5AC transcript and protein levels. Compared with control rats, smoke-exposed animals had significantly greater levels of MUC5AC in the bronchial epithelial cells after *P. aeruginosa* inoculation. The administration of Myrtol standardized markedly attenuated the upregulation of MUC5AC mRNA expression in lungs and MUC5AC protein expression in bronchiolar epithelia. These results strongly suggest that Myrtol standardized attenuates airway mucus hypersecretion induced by smoking and bacterial infection.

Increasing evidence suggests that cigarette smoke compromises the respiratory innate immune system. As a consequence, infectious agents may not be cleared effectively and bacterial colonization and prolonged inflammation may result. This can contribute to a decline in lung function and the development of COPD [21–25]. Bacterial clearance in the lower respiratory tract mainly depends primarily on MCC function and alveolar macrophages. It has been shown that smoking does not affect the phagocytic activity of macrophages [26]. As such, we hypothesized that MCC impairment was the primary cause for a reduced bacterial clearance in patients with COPD. Thus, inhibiting mucus overproduction may improve mucociliary transport and bacterial clearance.

*Pseudomonas aeruginosa* is an opportunistic pathogen that primarily infects patients with impaired host defenses [27] and is one of the primary infectious agents in patients with COPD [28, 29]. In the present study, we used *P. aeruginosa* to infect COPD rats and investigated the effects of Myrtol standardized on bacterial clearance. We observed a lower rate of bacterial clearance in cigarette smoke-exposed animals compared with non-smoke-exposed animals after *P. aeruginosa* infection. Myrtol standardized markedly attenuated levels of bacteria in the lungs of smoke-exposed rats 72 h after inoculation. *P. aeruginosa* colonizes in the lower respiratory tract by the combination of adhesions and mucins [30, 31]. Thus, mucus hypersecretion in the respiratory tract may promote bacterial colonization. It is a possible mechanism that Myrtol standardized may reduce bacterial adherence and multiplication in the airways by...
inhibiting MUC5AC expression and mucus hypersecretion. We note that the decreased amount of mucus in the airways may enhance ciliary movement and the MCC function, which aid bacterial clearance. In subsequent experiments we intend to investigate the mechanisms by which Myrtol standardized affects mucociliary transportation in more detail.

Cigarette smoke can stimulate the release of the pro-inflammatory factors TNF-α, IL-1β, IL-6 and IL-8 from monocytes and macrophages. It has been demonstrated that TNF-α can induce the increase in expression of airway mucus [32, 33]. A recent study in vitro demonstrated that 1,8 cineole affects the immune system and anti-inflammatory processes, and can decrease the release of pro-inflammatory factors such as TNF-α and IL-1β [9]. According to our study, Myrtol standardized reduces levels of the inflammatory factors TNF-α and IL-6 that are up-regulated by exposure to smoke and P. aeruginosa infection. Based on our findings, we suggest that the Myrtol standardized induced attenuation of mucus hypersecretion may be mediated through reducing the release of inflammatory cytokines in the lungs.

In conclusion, our study demonstrates that Myrtol standardized can promote both bacterial clearance in smoke-induced obstructive lungs infection and anti-inflammatory activity in the airway mucosa. It therefore can be considered as an alternative or supplementary treatment option in patients with acute or chronic airway inflammation and/or infection.

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Conflict of Interest
The authors have no conflicts of interest to declare. There were no financial links or other support with manufacturers of material or devices described in the paper.

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