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Research Article

Matrine reduces cigarette smoke-induced airway neutrophilic inflammation by enhancing neutrophil apoptosis

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Chronic Obstructive Pulmonary Disease (COPD) is a major incurable global health burden and will become the third largest cause of death in the world by 2030. It is well established that an exaggerated inflammatory and oxidative stress response to cigarette smoke (CS) leads to, emphysema, small airway fibrosis, mucus hypersecretion, and progressive airflow limitation. Current treatments have limited efficacy in inhibiting chronic inflammation and consequently do not reverse the pathology that initiates and drives the long-term progression of disease. In particular, there are no effective therapeutics that target neutrophilic inflammation in COPD, which is known to cause tissue damage by degranulation of a suite of proteolytic enzymes including neutrophil elastase (NE). Matrine, an alkaloid compound extracted from *Sophora flavescens Ait*, has well known anti-inflammatory activity. Therefore, the aim of the present study was to investigate whether matrine could inhibit CS-induced lung inflammation in mice. Matrine significantly reduced CS-induced bronchoalveolar lavage fluid (BALF) neutrophilia and NE activity in mice. The reduction in BALF neutrophils in CS-exposed mice by matrine was not due to reductions in pro-neutrophil cytokines/chemokines, but rather matrine's ability to cause apoptosis of neutrophils, which we demonstrated *ex vivo*. Thus, our data suggest that matrine has anti-inflammatory actions that could be of therapeutic potential in treating CS-induced lung inflammation observed in COPD.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a major incurable disease and has been predicted to be the third leading cause of deaths worldwide by 2030 [1]. COPD and its associated comorbidities are a cause of much mortality and morbidity, and a major economic burden on society, costing the U.S.A. approximately U.S. $50 billion in 2010 [2]. COPD is characterized by chronic airway inflammation, persistent respiratory symptoms, and irreversible airflow limitation due to airway and/or alveolar abnormalities, which are caused by significant exposure to noxious particles or gases [3]. Cigarette smoking is the major cause of COPD and has been shown to increase the mortality rate in patients with COPD compared with COPD patients who do not smoke [4]. The disease usually is progressive and after removal of the noxious particles, the inflammation can continue in a self-sustaining manner.

Unfortunately, current therapies are not effective in relieving the symptoms, decline in lung function, disease progression, and mortality in COPD because they do not suppress the underlying inflammation [5,6]. Moreover, the development of effective treatments for COPD have been severely hampered as the

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mechanisms and mediators that drive the induction and progression of chronic inflammation, emphysema, altered lung function, defective lung immunity, and many extrapulmonary comorbidities are still poorly understood [7]. Thus, a better understanding of the inflammatory and destructive processes in the pathophysiology of COPD will aid in the identification of therapeutic targets. Recent advances have included CXC chemokine receptor 2 (CXCR2) antagonists that block pulmonary neutrophil and monocyte recruitment, broad-spectrum anti-inflammatory drugs which include inhibitors of the proinflammatory enzymes phosphodiesterase-4, p38 mitogen-activated protein kinase, Janus kinases, NF-κB kinase, and PI3 kinase-γ and -δ, and reversal of corticosteroid resistance through increasing histone deacetylase-2 (HDAC2) activity [5,6].

Neutrophilic airway inflammation is a characteristic feature of COPD and contributes to the pathophysiology of disease [8]. Neutrophils are the most abundant white blood cells and play a central role during the innate immune response to protect the body against invading pathogens [9,10]. Neutrophils eliminate microbes through the process of phagocytosis and oxidative burst caused by the activation of NADPH oxidase, in which microorganisms are oxidized by electron transport and finally killed [11,12]. Beyond phagocytosis, neutrophils employ neutrophil extracellular traps (NETs) to capture microbes [13] that can be engulfed. In COPD patients, the population of neutrophils is kept at a higher level in the airways and blood than in healthy people [14]. The persistent neutrophilic inflammation is due to repeated infection and impaired innate immune functions [14,15]. The large influx of neutrophils results in enhanced release of proteases and mediators (e.g. oxidants) that destroy lung tissue and promote inflammation, respectively [16,17].

We have previously shown that the recruitment of neutrophils in response to cigarette smoke (CS) is dependent on a complex cytokine/chemokine network involving CSF2 (GM-CSF) and interleukin (IL) 17A (IL-17A) [18–22]. The recruitment of neutrophils is increased during infective acute exacerbations of COPD (AECOPD), where the influx of leukocytes fails to eradicate respiratory pathogens. Other strategies to block neutrophil recruitment in chronic lung diseases such as COPD have also been trialed. CXCR2 is a key receptor in the chemotaxis of neutrophils to sites of tissue inflammation, injury, and infection. ELR+ chemokines including IL-8, CXCL1, and CXCL2 recruit neutrophils through interaction with the G-protein-coupled CXCR1 and CXCR2 expressed on neutrophils. ELR+ chemokines that promote recruitment of neutrophils via CXCR2 are known to be elevated in COPD and clinical trials have trialed CXCR1/2 antagonists with limited benefit. In a more recent trial, the MK-7123 CXCR2 antagonist was used in COPD, which reduced sputum neutrophils and significantly improved lung function [23]. However, the compound was also associated with a reduction in absolute neutrophil count in blood, which may increase susceptibility to infection. Hence, alternative strategies to alleviate neutrophilic inflammation are needed.

It has been proposed that compounds which promote apoptosis of neutrophils can benefit inflammatory diseases [24]. Injection of apoptotic neutrophils can alleviate the inflammation induced by lipopolysaccharide (LPS) [25], so promoting the apoptosis of neutrophils and keeping neutrophil homeostasis represent a potential therapeutic target in the suppression of inflammation. However, only a few of the currently available medical therapies selectively target neutrophils.

Matrine is one of the active components of *Sophora flavescens* Ait (Kushen) which is currently being used to treat liver viral infection and tumors in China [26,27]. In previous studies, matrine has shown anti-inflammatory and antioxidative effects in acute lung injury and intestinal inflammation induced by LPS [28,29]. A study by Zeng et al. [30] showed that matrine alleviated the inflammation in a mouse model of insulin resistance and hepatic steatosis by increasing the hepatic levels of heat shock protein HSP72. Matrine was also shown to be cytotoxic to tumor cells by activating the apoptosis pathway of tumor cells in different studies [31,32]. In a recent study, matrine decreased the number of neutrophils in acute lung injury in mice [33] and inhibited myeloperoxidase (MPO) activity [28]. Therefore, we proposed that matrine may relieve inflammation by inducing apoptosis of neutrophils.

In the present study, we examined whether matrine can reduce CS-induced lung neutrophilia in mice. Our group has developed an acute 4-day CS exposure model to explore the mediators and mechanisms involved in the induction of CS-induced lung inflammation which has relevance to the pathogenesis of COPD [34,35]. Identification of compounds that can reduce the damaging effects of neutrophils may have clinical implications for the treatment of COPD.

Methods

Animals

Specific pathogen-free BALB/c mice aged 8 weeks and weighing ∼22 g were obtained from the Animal Resources Centre Pty. Ltd. (Perth, Australia). The animals were housed at 20°C on a 12-h day/night cycle in sterile micro-isolators and fed a standard sterile diet of Purina mouse chow with water allowed *ad libitum*. The experiments

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described in this manuscript were approved by the Animal Ethics Committee of RMIT University (Application ID 1521) and conducted in compliance with the guidelines of the National Health and Medical Research Council of Australia on animal experimentation.

CS exposure

Mice were placed in 18-l perspex chamber (The Plastic Man, Huntingdale, Victoria, Australia) in a fume cupboard (Aircare Extraction Systems Limited, Clayton, Victoria, Australia) and exposed to CS generated from nine cigarettes per day for 4 days (Days 0–3), delivered three times per day at 10 a.m., 1 and 4 p.m. with three cigarettes spaced over 1 h, as previously described [20,21]. CS was generated in 60 ml tidal volumes over 10 s, by use of timed draw-back mimicking normal smoking inhalation volume and cigarette burn rate. The mean total suspended particulate mass concentration in the chamber containing CS was 420 $mg.m^{-3}$. Sham-exposed mice were placed in 18-l perspex chamber but did not receive CS. Commercially available filter-tipped Winfield Red cigarettes (manufactured by Philip Morris, Australia) of the following composition were used: 16 mg or less of tar, 1.2 mg or less of nicotine, and 15 mg or less of CO. Mice were weighed daily at approximately the same time each day.

Matrine treatment

Mice were treated once a day with matrine (100 $mg.kg^{-1}$) or vehicle (saline) via oral gavage 1 h before the first CS exposure for the day until they were killed after 4 days exposure to CS. The dose for matrine was based on our previously published studies [30]. In separate experiments, mice were also treated with matrine (10 and 30 $mg.kg^{-1}$) or vehicle (saline) 1 h before the mice were first exposed to CS each day for 4 days, via intranasal administration after the mice were anesthetized with methoxyflurane (Medical Developments International, Victoria, Australia). Mice were killed after the 4-day CS exposure. Matrine was purchased by Professor Jiming Ye from Sigma–Aldrich, Shanghai, China.

Bronchoalveolar lavage and lung collection

Animals were killed by i.p. injection of sodium pentobarbitone (240 $mg.kg^{-1}$) (Virbac, NSW, Australia) on day 4. Lungs were then lavaged *in situ* with a 400-μl aliquot of PBS, followed by three 300 μl aliquots as previously described [21]. In total up to 1 ml of bronchoalvealor lavage fluid (BALF) was retrieved per mouse. The total number of viable cells in the BALF was measured, cytospins were prepared, and cells were differentiated by standard morphological criteria as previously published [20,21]. Residual BALF was centrifuged to collect the supernatant for storage at -80°C. Whole lungs were perfused free of blood via right ventricular perfusion with 5 ml of PBS, rapidly excised *en bloc*, and snap-frozen in liquid nitrogen, and stored at -80°C until required.

Quantitative real-time PCR

Total RNA was extracted from approximately 15 mg of whole lung tissue from six to eight mice per treatment group using RNeasy Mini Kits (Qiagen, Germany), reverse transcribed with High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, MA, U.S.A.), and triplicate real-time PCR with Thermo Fisher Scientific pre-developed Taqman assay reagents were performed. *GAPDH* mRNA was used as the internal housekeeping control. The threshold cycle (C_t) value is the PCR cycle number (out of 40) at which the measured fluorescent signal exceeds a calculated background threshold identifying amplification of the target sequence value and is proportional to the number of input target copies present in the sample. C_t numbers were transformed with the $\Delta\Delta C_t$ (threshold cycle time) and relative value method and were expressed relative to *GAPDH* mRNA levels.

Measurement of neutrophil elastase activity and MPO activity

Neutrophil elastase (NE) activity in BALF as a marker for neutrophil activity was measured using an EnzChek™ Elastase Assay Kit (Thermo Fisher Scientific, MA, U.S.A.) according to the manufacturer's instructions. Briefly, 30 μl of BALF was diluted with 60 μl of 1× Reaction Buffer in a 96-well plate, to which 10 μl of 50 μg/ml DQ elastin substrate was added. Reaction was protected from light and incubated at 37°C overnight. Fluorescence was then measured using the CLARIOstar® plate reader (Ex/Em: 505/515 nm; BMG Labtech, Germany). MPO activity was assessed by homogenizing ground lung tissue (50 $mg.ml^{-1}$) in extraction buffer (50 mM potassium phosphate monobasic pH 6.0, 0.5% hexadecyltrimethyl ammonium bromide and 10 mM EDTA). Following centrifugation, lung lysate was incubated with reaction buffer (50 mM potassium phosphate monobasic pH 6.0, 0.167 $mg.ml^{-1}$ o-Da (Fast Blue B, Sigma), and 0.005% H_2O_2) and the change in absorbance at 460 nm per minute (2–3 min), resulting from decomposition of H_2O_2 and subsequent oxidation of o-Da, was measured using the CLARIOstar® plate reader.

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Neutrophil isolation and cell culture

Neutrophils were isolated from the bone marrow of untreated male Balb/c mice through negative selection using the MojoSort™ Mouse Neutrophil Isolation Kit (Biolegend, CA, U.S.A.) with the MidiMACS™ magnetic separator and LS Column (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. In brief, bone marrow cells were collected from the femur and tibia of Balb/c mice. The cells were incubated with the Biotin-Antibody Cocktail from the kit for 15 min on ice, followed by incubation with Strepavidin Nanobeads for another 15 min on ice. The cells were passed through an LS column in a MidiMACS™ magnetic separator and the flow-through containing bone marrow isolated neutrophils (BMN) were collected.

Preparation of CS extract

CS extract (CSE) was prepared as previously described, with a minor modification [36]. Briefly, one filtered cigarette (Winfield Red; Phillp Morris, Australia) was bubbled through 25 ml of RPMI 1640 medium supplemented with 10% FBS (Thermo Fisher Scientific, MA, U.S.A.). This preparation was filtered through a 0.2-μm filter and arbitrarily assigned a concentration of 100% CSE. For the apoptosis experiments, BMN were incubated in 5% CSE or vehicle (RPMI 1640 with 10% FBS) at 37°C for 24 h.

Culture of bone BMNs

BMNs were exposed to either control (RPMI 1640 with 10% FBS) or 5% CSE, along with various concentrations of matrine (0, 0.1, and 1 mM). The cells were incubated in 5% CO_2, at 37°C for 24 h after isolation from the bone marrow.

Apoptosis assay of BMNs

After 24 h, BMNs were stained using Annexin V-FITC/propidium iodide (PI) staining (BD Biosciences, Franklin Lakes, NJ, U.S.A.). Fluorescence quantitation was performed by flow cytometry using a FACSAria III sorter (BD Biosciences). Approximately one hundred thousand cells were recorded using the BD FACSDIVA software and subsequently analyzed using FlowJo 10 (BD Biosciences) software. The annexin V and PI double negative cells were defined as live cells. Cells stained with annexin V were early-stage apoptotic cells, and cells stained with both annexin V and PI were defined as late-stage apoptotic cells. The cells which were stained with PI alone were cells undergoing the necrosis process. The results were expressed as a percentage of total number of gated neutrophils.

Statistical analysis

Grouped data are expressed as mean ± S.E.M.; n represents the number of mice per treatment group. Differences were determined by two-way ANOVA followed by Sidak post-hoc test for multiple comparisons, where appropriate. All statistical analyses were performed using GraphPad Prism for Windows (version 7.02). Statistical significance was indicated by using $P<0.05$ or $P<0.01$.

Results

Matrine does not affect CS-induced body weight loss

The body weight of each mouse was measured daily and the difference in body weight was calculated as a percentage of their initial body weight just prior to the start of the CS exposure on day 0 (Figure 1). CS exposure caused a significant decrease in body weight over the 4-day CS exposure protocol ($P<0.05$, Figure 1). At the end of the experiment (day 4), the CS-exposed vehicle-treated lost approximately 5.4 ± 0.5% body weight compared with sham-exposed vehicle-treated mice (−1.1 ± 0.9%; $P<0.01$). Matrine had no effect on CS-induced weight loss when compared with CS-exposed vehicle mice (4.0 ± 0.5 and 5.4 ± 0.5%, respectively). There was also no significant difference in the mean percent weight change between sham mice treated with matrine (−2.9 ± 0.7%) and sham mice treated with the vehicle (−1.1 ± 1.0%).

Matrine reduces CS-induced BALF neutrophila

The cellular inflammatory response in the lung was examined by analyzing the BALF obtained from each mouse gavaged with either vehicle or matrine (Figure 2A–E). Vehicle-treated CS-exposed mice had significantly more total cells ($7.8 \pm 0.8 \times 10^5$), macrophages ($3.4 \pm 0.4 \times 10^5$) and neutrophils ($4.4 \pm 1.0 \times 10^5$) in BALF (Figure 2A–C) compared with sham-exposed mice treated with vehicle ($1.8 \pm 0.28 \times 10^5$, $1.8 \pm 0.2 \times 10^5$, $1.4 \pm 1.0 \times 10^2$; $P<0.05$). Oral administration of matrine (100 $mg.kg^{-1}$) in CS-exposed mice did not alter the total number of cells (6.2 ± 0.7

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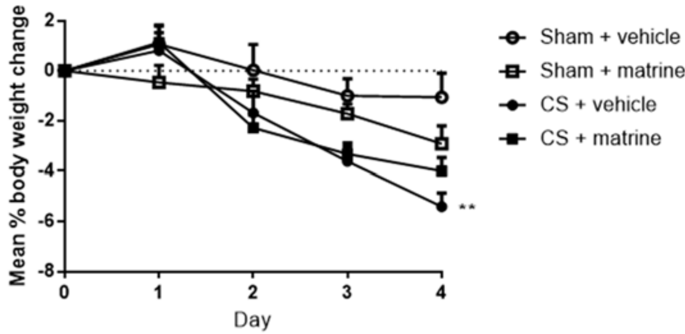

Figure 1. Effect of matrine (100 mg.kg^{-1}, oral gavage) on CS-induced body weight loss
Mouse body weights are shown as % body weight change and expressed as mean ± S.E.M. for n=8 mice per treatment group and are representative of two independent experiments. Mice were treated with matrine (100 mg.kg^{-1}) or vehicle (saline) by oral gavage 1 h before the first daily CS exposure. Two-way ANOVA with Sidak post-hoc test was performed to assess statistical significance **P<0.01, compared with sham+vehicle mice.

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× 10^5) and macrophages (3.6 ± 0.6 × 10^5) but did significantly decrease the number of neutrophils (2.6 ± 0.4 × 10^5) and lymphocytes (1.3 ± 1.2 × 10^2; Figure 2D) in the BALF compared with CS-exposed mice treated with vehicle (lymphocyte: 25.1 ± 10.2 × 10^2; P<0.05). Matrine did not have any effect on the total number of cells (1.7 ± 0.3 × 10^5), macrophages (1.7 ± 0.3 × 10^5), neutrophils (2.5 ± 0.9 × 10^2) and lymphocytes (3.1 ± 1.8 × 10^2) in sham mice compared with vehicle-treated sham mice (lymphocyte: 7.4 ± 4.5 × 10^2).

Mice were also intranasally administered vehicle or matrine (10 and 30 mg.kg^{-1}) each day 1 h prior to the first CS exposure session each day for 4 days (Figure 3A–E). This delivery method allows direct instillation into the lungs. The BALF from CS-exposed mice treated with vehicle contained significantly more total cells (9.7 ± 0.7 × 10^5), macrophages (2.0 ± 0.2 × 10^5), and neutrophils (9.3 ± 0.6 × 10^5) than sham-exposed mice treated with vehicle (1.7 ± 0.4 × 10^5, 1.5 ± 0.2 × 10^5, 3.2 × 10^3, Figure 3A–C). CS-exposed mice treated intranasally with 10 and 30 mg.kg^{-1} matrine showed no significant difference in the total number of cells in the BALF (10.6 ± 0.9 × 10^5 and 9.7 ± 0.7 × 10^5, respectively) when compared with CS-exposed mice treated with vehicle. However, CS-exposed mice treated with 30 mg.kg^{-1} matrine had significantly more macrophages (3.3 ± 0.5 × 10^5) and significantly less neutrophils (6.3 ± 0.4 × 10^5) when compared with the CS-exposed mice treated with the vehicle. There was no significant difference in macrophage (3.0 ± 0.4 × 10^5) or neutrophil (7.6 ± 0.6 × 10^5) populations in the BALF of CS-exposed mice treated with 10 mg.kg^{-1} matrine when compared with CS-exposed mice treated with vehicle. There was no significant difference in lymphocytes in the BALF amongst the four groups of mice (Figure 3D).

Matrine did not decrease pro-neutrophilic cytokines or other cytokines in CS-exposed mice

To identify which mediators drive the airway inflammatory response, in particular the very significant alleviation of neutrophils, during CS exposure mice were treated with matrine by oral gavage and mRNA levels of key inflammatory cytokines, chemokines, and proteases implicated in airway inflammation were measured in whole lung tissue by quantitative real-time PCR (QPCR).

In vehicle-treated mice, exposure to CS for 4 days significantly increased the mRNA expression of cytokines (CSF2 [GM-CSF], TNFα, IL-6, IL-17A, IL-1β), chemokines (CXCL1, CXCL2, CCL2), and matrix metallopeptidase (MMP9, MMP12) (P<0.01; Table 1) compared with their sham counterparts. We also observed that sham mice treated orally with matrine (100 mg kg^{-1}) had a small but statistically significant increase (∼46%) in mRNA expression of IL-1β in the lung tissue compared with sham mice treated with vehicle. mRNA expression of neutrophil attractant CXCL1 and matrix metalloproteinase MMP12 in lung tissue was significantly increased (46 and 22%, respectively) in the CS-exposed mice treated with matrine compared with CS-exposed mice treated with vehicle. The level of mRNA expression of pro-neutrophil cytokine IL-17A appeared to increase, but was not statistically significant, in CS-exposed mice treated with matrine than in CS-exposed mice treated with vehicle.

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Figure 2. Effect of matrine (100 mg.kg^{-1}, oral gavage) on neutrophils and lymphocytes in BALF of mice exposed to CS for 4 days

BALF cellularity is shown as (**A**) the total number of cells, (**B**) macrophages, (**C**) neutrophils, and (**D**) lymphocytes. Data are expressed as mean ± S.E.M. for n=6–8 per treatment group and are representative of two independent experiments. (**E**) Representative DiffQuik-stained cytospin preparations of BALF from sham- and CS-exposed mice treated with either vehicle or matrine (100 mg.kg^{-1}, oral gavage). Two-way ANOVA with Sidak *post hoc* test was performed to assess statistical significance. *$P<0.05$, **$P<0.01$, ****$P<0.0001$, $^{\#}P<0.05$.

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Figure 3. Effect of intranasal administration of matrine on BALF cellularity in mice exposed to CS for 4 days

BALF cellularity is shown as (**A**) total number of the cells, (**B**) macrophages, (**C**) neutrophils, and (**D**) lymphocytes. Data are expressed as mean ± S.E.M. for n=7–10 per treatment group and are representative of two independent experiments. (**E**) Representative DiffQuik-stained cytospin preparations of BALF from sham- and CS-exposed mice treated with either vehicle or matrine (10 and 30 mg.kg^{-1}, intranasally). One-way ANOVA with Sidak *post hoc* test was performed to assess statistical significance. $^{****}P<0.0001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$.

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Table 1 Effect of oral administration of matrine on whole lung cytokine, chemokine, and protease mRNA expression in CS-exposed mice

Gene	Treatment			
	Sham		CS	
	Vehicle	Matrine	Vehicle	Matrine
Cytokines				
GM-CSF	1.05 ± 0.14	0.88 ± 0.04	2.49 ± 0.13[2]	2.68 ± 0.15
TNFα	1.03 ± 0.1	1.04 ± 0.13	3.25 ± 0.21[2]	3.65 ± 0.26
IL-6	1.03 ± 0.09	0.87 ± 0.10	3.06 ± 0.20[2]	3.04 ± 0.20
IL-17A	1.02 ± 0.12	2.49 ± 0.62	4.48 ± 0.44[2]	6.97 ± 1.14
IL-1β	1.02 ± 0.07	1.49 ± 0.15[1]	2.34 ± 0.05[2]	2.77 ± 0.15
Chemokines				
CXCL1	1.06 ± 0.14	1.69 ± 0.19	16.43 ± 1.90[2]	24 ± 2.15[4]
CXCL2	1.1 ± 0.22	1.15 ± 0.17	12.17 ± 1.00[2]	11.87 ± 1.02
CCL2	1.02 ± 0.08	1.15 ± 0.13	11.15 ± 0.53[2]	12.5 ± 0.35
Matrix metalloproteinase				
MMP9	1.09 ± 0.17	1.08 ± 0.10	1.58 ± 0.09[2]	1.84 ± 0.07
MMP12	1.06 ± 0.15	0.75 ± 0.05	3.79 ± 0.12[2]	4.61 ± 0.20[3]

mRNA expression for all genes was measured simultaneously under identical conditions using QPCR. Responses are shown as fold-change relative to sham+vehicle mice after normalization to GAPDH housekeeping mRNA expression. Data are shown as the mean ± S.E.M. for triplicate reactions of six to eight individual mouse lungs from one experiment. Two-way ANOVA with Sidak post hoc test was performed to assess statistical significance.
[1]$P<0.01$ and [2]$P<0.05$, compared with sham+vehicle mice.
[3]$P<0.05$ and [4]$P<0.01$ compared with CS+vehicle mice.

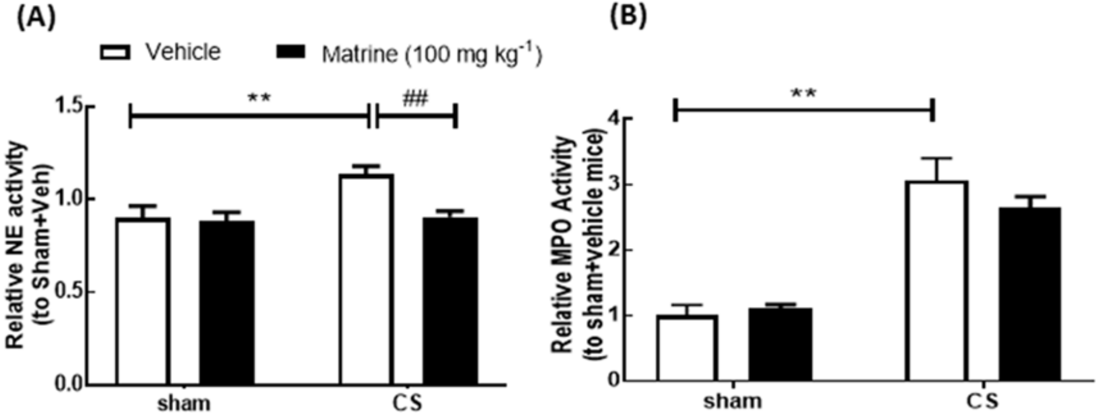

Figure 4. Effect of oral administration of matrine (100 mg.kg^{-1}) on NE and MPO activity in mice exposed to CS for 4 days
(**A**) NE activity in BALF and (**B**) MPO activity in lung tissue. Data are shown as the mean ± S.E.M. for one reaction of seven to eight individual mouse lungs from one experiment. Two-way ANOVA with Sidak *post hoc* test was performed to assess statistical significance. $^{**}P<0.01$, $^{\#\#}P<0.05$.

Impact of matrine on NE and MPO activity in CS-exposed mice

To determine if the two important neutrophil markers, NE and MPO, were also reduced by matrine treatment, we performed assays to measure the activity of NE in BALF and MPO activity in lung tissues obtained from these mice (Figure 4). After 4 days of CS exposure, the vehicle-treated mice had significantly increased MPO activity (3.06 ± 0.35) and NE activity (1.13 ± 0.05) compared with the vehicle-treated mice in the sham group (1.00 ± 0.16, 0.90 ± 0.06) ($P<0.05$). Matrine administration reduced NE activity in BALF of CS-exposed mice (0.91 ± 0.03) compared with their vehicle-treated counterpart. MPO activity in lung tissue was not statistically significant in CS-exposed mice treated with matrine when compared with CS-exposed mice treated with vehicle.

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Matrine reduces CS-induced neutrophilia by promoting the apoptosis of the neutrophils

As the reduction in neutrophils in the BALF of CS-exposed mice treated with matrine did not correlate with the lack of reduction in pro-neutrophil cytokines/chemokines, we then went on to explore whether the reduction in neutrophils by matrine was due to increased apoptosis. Hence, neutrophils isolated from the bone marrow of untreated male Balb/c mice were cultured in the presence of either control medium (RPMI 1640 + 10% FBS) or 5% CSE, in the presence or absence of various concentrations of matrine (0, 0.1, and 1 mM) for 24 h. Apoptotic state of the cells was determined through flow cytometry on the neutrophils stained with Annexin V-FITC and PI.

Matrine alone caused the majority of neutrophils to enter apoptosis especially at the higher concentrations of 1 mM compared with cells in control medium with no matrine (Figure 5). Cells incubated in 0.1 mM of matrine were mostly in the early apoptotic state, while cells incubated in 1 mM matrine were mostly in the late apoptotic state after 24 h. Matrine did not promote neutrophils to enter the necrotic pathway.

Most neutrophils incubated in CSE for 24 h underwent cell death and had entered the apoptotic state, either in the early phase or the late phase of apoptosis (Figure 5). Due to this, matrine was unable to exert an increased apoptotic effect on neutrophils incubated in CSE and matrine since most cells were dead. CSE did not cause neutrophils to enter the necrotic pathway.

Discussion

Airway inflammation is a characteristic feature of COPD and contributes to the pathophysiology of COPD through the release of proteases and mediators which serve to destroy lung tissue and perpetuate the inflammatory response. COPD is a progressive condition and after removal of the noxious particles (i.e. CS), the inflammation can continue in a self-sustaining manner. Thus, resolution of inflammation is key to treat COPD. In the present study, we tested the hypothesis that matrine could suppress inflammation in the airway of mice exposed to CS based on its anti-inflammatory effect in other inflammatory diseases [29,37]. In our *in vivo* and *ex vivo* studies, we found that matrine dampened airway inflammation by reducing the influx of neutrophils but not macrophages in the airways of CS-exposed mice. Importantly, the pharmacological effect of matrine on neutrophils was not the result of down-regulation of pro-neutrophil attractant expression in the lung tissue, such as CXCL1, but rather matrine causing neutrophils to undergo apoptosis.

Matrine is a small molecular compound which is extracted from the traditional Chinese herb *Sophora flavescens* Ait [38]. *Sophora flavescens* Ait is used to treat viral hepatitis and other various acute inflammatory diseases in clinics across China [26,27]. Matrine and its different derivatives were reported to have multiple effects in different disease models. For example, matrine can prevent bone loss [38], protect glial cells from central nervous system inflammation [39], alleviate intestinal inflammation [29], and decrease liver cell injury [40]. Additionally, it is reported that matrine showed very strong anti-tumor effect which includes cytotoxic effect on prostate tumor cells, bladder tumor cells, and breast cancer cells [31,41,42]. All of the above observations are based on matrine's anti-inflammatory effect and ability to induce apoptosis. In the present study, CS-exposed mice treated with matrine had lower numbers of neutrophils in the airways, which demonstrates that matrine can alleviate airway inflammation in our acute CS exposure model. This result is similar to a previous study showing that matrine can reduce LPS-induced lung inflammation [28].

Many drugs that reduce the number of neutrophils in inflammatory settings often do so by down-regulating the expression of neutrophil chemoattractants [43,44]. A recent study showed that depletion of CXCL1 attenuated neutrophil recruitment, reduced the histology score and bacteria clearance [45], thus demonstrating that down-regulation of neutrophil attractants is an attractive target in resolving inflammation. We have also previously shown that neutralizing the chemoattractant IL-17A can reduce CS-induced BALF inflammation [19]. However, in this study matrine did not decrease the expression of neutrophil chemoattractants, such as CXCL2 and IL-17A, in CS-exposed mice, and in fact increased the expression of CXCL1. Thus, the reduction in the number of neutrophils by matrine does not seem to be due to matrine suppressing neutrophil recruitment. The elevated expression of neutrophil chemoattractant CXCL1 and the reduction in neutrophils in the lungs suggests that the negative feedback mechanism is still trying to recruit neutrophils to the lungs. We suspect that neutrophils may be dying before recruitment into the lungs in CS-exposed mice treated with matrine, causing continued up-regulation of neutrophil attractants in the lungs to try and recruit more neutrophils.

To verify the killing effect of matrine, we administered matrine instranasally into the lungs of CS-exposed mice and counted the cell populations in the BALF. Compared with the CS-exposed mice, the number of neutrophils was reduced by approximately 18 and 29% with 10 and 30 mg/kg, respectively. However, the number of macrophages were

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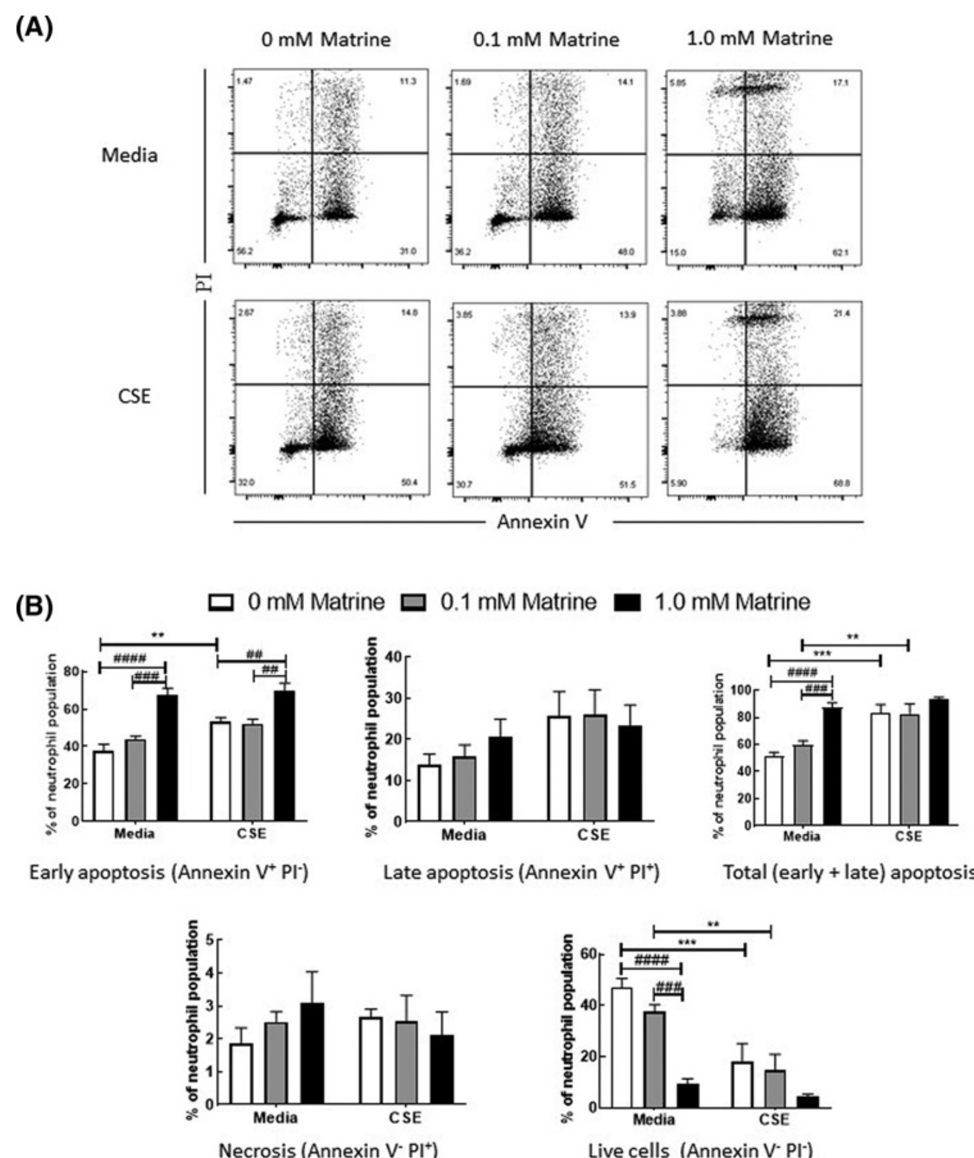

Figure 5. Effect of matrine on apoptosis of BMNs

BMNs were treated with matrine (0, 0.1, and 1.0 mM) with/without CSE for 24 h, followed by Annexin V and PI staining. (**A**) A representative flow cytometric plot of Annexin V and PI staining of a single sample from each treatment group. The percentages of neutrophils in each quadrant are shown. Live cells were considered to be Annexin V− PI−, early apoptotic cells were Annexin V+ PI−, late stage apoptotic cells were Annexin V+ PI+ and necrotic cells were considered to be Annexin V− PI+. (**B**) Graph of pooled data of the different stages of cell death and total (early + late) apoptotic cells. Data are expressed as mean ± S.E.M. for n=3–4 per treatment group from three to four separate experiments. Two-way ANOVA with Sidak test was performed. P-value represents $^{**}P<0.01$, $^{***}P<0.001$, $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.001$.

increased by approximately 52 and 65% at the 10 and 30 mg/kg doses, respectively. This increase in macrophages may be a defense mechanism whereby macrophages are recruited to the lung to help clear the neutrophils killed by matrine.

NE, the main enzyme in neutrophils, is often used as a marker of neutrophils. It also plays a critical role in emphysema induced by CS [46]. NE activity was higher in the BALF of CS-exposed mice compared with vehicle-treated sham-exposed mice presumably because of the higher number of neutrophils in the BALF in response to CS. Importantly, NE activity was reduced in the BALF of matrine-treated CS-exposed mice compared with vehicle-treated CS-exposed mice and this was likely due to the reduced numbers of BALF neutrophils. Moreover, matrine did not

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decrease the rate of necrotic cells in CSE-treated neutrophils, which suggests that matrine might not dampen the release of NE.

It has been reported that CSE and nicotine can delay the spontaneous death of neutrophils by suppressing apoptosis [47,48], which indicates that CS can alter the normal lifespan of neutrophils. However, the prolonged lifespan of neutrophils does not enhance host immunity but contributes to bacterial persistence in the lungs of smokers and is likely to promote further pulmonary recruitment of neutrophils. Not all studies found that CSE suppressed the apoptotic course of neutrophils and that some suggest that CSE could induce apoptosis of neutrophils [49]. In the present study, we found that CSE did indeed induce apoptosis of neutrophils and that 1 mM matrine induced most neutrophils to enter early apoptosis after being incubated for 24 h in culture medium or CSE. This result is consistent with the observations from our *in vivo* experiments where intranasal administration of 30 $mg.kg^{-1}$ matrine caused a reduction in neutrophils in CS-exposed mice.

Externalization of phosphatidylserine is a characteristic of the early-stage apoptosis process [50], being a signal to regulate the recognition and engulfment of apoptotic cells by macrophages [51]. Removal of neutrophils by macrophages is beneficial for the resolution of inflammation. After 30 $mg.kg^{-1}$ matrine was intranasally administered into the airways of the CS-exposed mice, more macrophages were detected in the airway compared with the CS-exposed mice. However oral administration of matrine alone did not increase the number of macrophages in the airway. Thus, we further examined the expression of CCL2, an attractant of macrophages, and found that it was not changed by matrine treatment. This implies that macrophage recruitment may be due to the apoptosis of neutrophils induced by matrine. Indeed, we found the mRNA expression of MMP12, a marker of macrophages, was elevated in CS-exposed mice after oral administration of matrine. These observations were evidence that more macrophages had infiltrated the pulmonary mesenchyme.

The present study also found that oral administration of matrine reduced the number of lymphocytes in airway. However, in airway of the mice, the number of lymphocytes was not increased by CS exposure, which is accordant with previous studies performed by our group [21,52]. In addition, the number of lymphocytes was not changed by intranasal instillation of matrine.

Conclusion

The present study showed that matrine can reduce CS-induced neutrophilic inflammation by inducing neutrophil apoptosis. Therefore, matrine may be a potentially beneficial compound to treat neutrophilic inflammatory diseases including COPD by targetting excessive neutrophilic inflammation in the airway.

Clinical perspectives

- CS is the major cause of COPD. Current treatments have limited efficacy in inhibiting inflammation and fail to modify the factors that initiate and drive the long-term progression of disease. Matrine is a small molecule which has well-known anti-inflammatory activity. The aim of the present study was to investigate whether matrine could inhibit CS-induced lung inflammation *in vivo*.

- The results of the present study show that matrine significantly reduced CS-induced BALF neutrophilia by causing apotosis of neutrophils and not by reducing pro-neutrophil cytokine/chemokine expression.

- Our data suggest that matrine may be an effective anti-inflammatory treat CS-induced BALF neutrophilia observed in COPD.

Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Author contribution

R.V. and J.-M.Y conceived the study design. X.Y., S.B. and R.V. designed the experiments. X.Y., H.J.S., H.W. and D.A. acquired the data. X.Y., H.J.S., H.W., D.A., J.Y., S.B., L.L. and R.V. analysed and interpreted the data. X.Y., J.-M.Y., S.B. and R.V. drafted the manuscript. H.W., D.A., H.J.S. and L.L. reviewed the manuscript and provided comments.

Abbreviations

BALF, bronchoalveolar lavage fluid; BMN, bone marrow isolated neutrophil; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; CSE, CS extract; C_t, threshold cycle; CXCR2, CXC chemokine receptor 2; IL, interleukin; LPS, lipopolysaccharide; MPO, myeloperoxidase; NE, neutrophil elastase; PI, propidium iodide; QPCR, quantitative real-time PCR.

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