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Effects of different corticosteroid doses and durations on smoke inhalation-induced acute lung injury and pulmonary fibrosis in the rat☆

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ABSTRACT

Excessive inflammation induced by cytokine storm and coagulation disorders is considered the primary characteristic of smoke inhalation-induced acute lung injury (SI-ALI). Glucocorticoids such as methylprednisolone (MP) are commonly used to treat patients with inflammatory diseases; however, the management of ALI or acute respiratory distress syndrome (ARDS) remains controversial. We explored the effects of different MP doses and durations in a rat SI-ALI model. SI-ALI model rats had a high mortality rate and severe lung injury with proinflammatory, procoagulant, and pro-fibrotic changes. We found that a medium MP dose (4 mg/kg) markedly improved survival rates compared with low (0.4 mg/kg) and high (40 mg/kg) doses in the acute phase. A medium dose significantly attenuated lung injury, and reduced proinflammatory cytokine production and neutrophil infiltration into alveoli. Both medium and high MP doses improved coagulation and fibrinolysis conditions compared with low-dose MP. We also explored the effect of different durations of MP treatment on attenuating fibrotic changes in late-phase SI-ALI. Pro-fibrotic chemokine levels were gradually increased, followed by an increase in collagen and fibrin deposition after smoke inhalation. Three and 7-day MP treatments significantly attenuated this process, which was reflected by a reduction in pro-fibrotic chemokine levels. There was no significant difference between 3- and 7-day treatments. We report that a medium MP (4 mg/kg) dose significantly reduced inflammation and coagulation disorders, as well as acute-phase mortality. Three-day MP treatment may sufficiently attenuate fibrotic changes in late-phase SI-ALI.

1. Introduction

Pulmonary injury resulting from acute smoke inhalation, which accounts for about 12% of patients in burn units, is the main cause of early death [1] and contributes significantly to the overall morbidity and mortality of fire victims [2]. There is a lack of uniform criteria for the diagnosis and definition of smoke inhalation-induced acute lung injury (SI-ALI), and, despite extensive research, mortality rates have changed little in recent decades [1].

SI-ALI differs from other kinds of lung injuries characterized by sudden onset and complicated insult elements, such as micro-particle deposition, systemic or pulmonary toxicity caused by irritant gases, or thermal injury above the glottis. The formation of reactive oxygen and nitrogen species, as well as the pro-coagulant and anti-fibrinolytic imbalance of alveolar homeostasis, contribute greatly to the pathogenesis of smoke inhalation injury [3,4].

Profound inflammation in airways with pulmonary shunting and an increased micro-vascular pressure gradient often result in acute hypoxemic respiratory failure, which is modulated by an increased inflammatory response [5,6], increased pulmonary leukocyte activity, and pronounced alveolar neutrophilia [7]. Large numbers of activated neutrophils damage alveolar epithelial cells (AECs) by releasing toxic molecules that cause the dissolution of tight junctions in AECs, apoptosis of AECs, and necrosis of alveolar type I (ATI) and alveolar type II (ATII) cells [8].

Inhalation trauma is associated with proinflammatory and procoagulant disturbances in the alveoli, as demonstrated in patients with ALI [9,10]. In addition, excessive fibrin and collagen deposition [4,11] leads to the chronic phase of respiratory disorders and hypoxemia [12]. A severe imbalance in systemic and alveolar hemostasis plays a central

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role in ALI and represents an independent risk factor for mortality of ALI patients [13]. Antithrombin III (ATIII), thrombin-antithrombin complex (TATc), and plasminogen activator inhibitor 1 (PAI-1) were proposed as biologic markers of fibrinolysis and inflammation because they are significantly associated with patient morbidity and mortality [14]. These markers are elevated in lung lavage fluid from burn patients with inhalation trauma, and they correlate with markers of inflammation and coagulopathy. The interaction between activated neutrophils, endothelial cells, and coagulative proteases is involved in the complex process of coagulation disturbance [15]. Excessive production of these factors leads to the deterioration of large and small airways within hours and causes delayed injury after smoke inhalation. Consequently, treatments that inhibit the inflammatory and procoagulation processes and enable the effective treatment of these patients are of great value.

Glucocorticoids (GCs) are potent anti-inflammatory drugs that act primarily by binding to cytoplasmic GC receptors. GC-receptor complexes bind to specific DNA motifs (glucocorticoid response elements, GREs) to transactivate gene transcription, or tether to DNA-bound transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and AP-1, which modulate the transcription of many proinflammatory cytokines (e.g., interleukin (IL)-6, IL-17a, and tumor necrosis factor (TNF)-α) [16]. Additionally, GCs act synergistically with natural anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IL-1 receptor antagonist [17].

Because GCs have potent anti-inflammatory effects, they might be effective for the treatment of patients with acute respiratory distress syndrome (ARDS). Although short-term high-dose GC therapy is considered ineffective in ARDS patients [18], low-dose, long-term use of corticosteroid therapy has been reported to be effective because it provides a continual inhibition of the systemic inflammatory response syndrome (SIRS) that accompanies ARDS [19–21]. Additionally, GC treatment in the early phase of lung inflammation resolved ALI and ARDS [22–24]. Rocco and colleagues [25] observed that an early, single corticosteroid dose adequately regulated the remodeling process in paraquat-induced ALI, resulting in improved lung mechanics. A larger clinical study is currently investigating the parameters for more effective GC treatment to develop appropriate guidelines (clinical trial NCT01731795).

Regarding interstitial lung diseases such as idiopathic pulmonary fibrosis (IPF), the administration of GCs or immunosuppressive agents to address diagnostic uncertainty and to suppress proinflammatory and profibrotic pathways is a conventional strategy for the treatment of IPF patients. When GCs are combined with pirfenidone, cyclophosphamide, or azathioprine, their potency was increased and a significant survival advantage was achieved [26–29]. Glucocorticoids also affect fibrotic pathways, inhibiting fibroblast proliferation and decreasing collagen deposition [16]. However, because of observed side effects, the use of GCs in SI-ALI remains controversial. The empirical administration of GCs is not recommended for this type of trauma, regardless of their acute-phase anti-inflammatory properties [30]. The main challenge is that the risk of infection caused by increased debris in the airway, and which reduces clearance efficiency and immunosuppression in response to GC treatment, increases two-fold [31].

Thus, we compared the therapeutic efficacy of MP, a GC, at different doses and durations for the treatment of SI-ALI and pulmonary fibrosis, to determine the optimal dosing regimen and schedule. This treatment protocol is guided by the pathogenesis of fibrosis and documented preclinical data, which showed that inflammation plays a major role in SI-ALI pathogenesis.

2. Materials and methods

2.1. Reagents

Polyurethane foam, rubber foam, acrylic ester, wood, and silicone acrylic latex paint, which are the most common smoke producers in modern fire disasters, were used as composite combustion materials. These materials caused cytoplasmic vacuolization, cytoplasmic blebbing, and hyperplasia in the distal lung as well as tracheal vacuolization [32–34].

Methylprednisolone (MP, Pfizer, Belgium) and pentobarbital sodium (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in normal saline (NS). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-17a, and tumor necrosis factor α (TNF-α) were obtained from R&D Systems (Minneapolis, MN, USA). ELISA kits for PAI-1, TATc, and ATIII were obtained from GENXSPAN (AL, USA). The following primary antibodies were used: anti-IL-10, anti-myeloperoxidase (MPO), anti-high mobility group box 1 (HMGB1), and anti-connective tissue growth factor (CTGF). All antibodies were purchased from Abcam (Abcam, Cambridge, MA, USA). The bicinchoninic acid (BCA) protein assay and DNA fragmentation detection kits were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA).

2.2. Animal care and use

Sprague-Dawley (SD) rats weighing between 200 and 220 g were provided by the animal facility at the Academy of Military Medical Sciences (Beijing, China). All rats were housed in polypropylene boxes, maintained at 22 ± 2 °C with a 12: 12 h light-dark cycle, and they had free access to food and water. All animal procedures were performed in accordance with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (publication no. 96-01). The Institutional Animal Care and Use Committee of the Naval General Hospital, People's Republic of China, approved all experiments and animal care procedures.

2.3. Smoke inhalation-induced lung injury models

The smoke inhalation-induced lung injury model was established based on the method described by Zhu et al. [11]. Briefly, 100 g of mixed materials (20 g of each material) were placed into a pot on a plate in a smoke-generating box, and the materials were then heated to produce smoke. Using an air blower, the smoke was delivered from the box to a 40 × 40 × 50 cm chamber through a smoke extractor pipe (30 cm in length and 20 cm in diameter). This chamber was maintained at a temperature of 24–27 °C with a water circulation cooler system to ensure that thermal injury of the smoke was excluded.

The concentration of oxygen (O_2), carbon monoxide (CO), and hydrogen sulfide (H_2S) was detected using an EM-4 L gas detector (Australia New Meter Group, Hong Kong, China). The gas detector and air blower were used to maintain a steady smoke environment in the chamber, with O_2 maintained between 18 and 20%, CO between 450 and 500 ppm, and H_2S between 5 and 10 ppm. When the gas concentrations were below the ranges mentioned above, the air blower would start to deliver smoke from the box to the chamber. After the chamber was filled with smoke, rats were placed in the chamber for 30 min. During exposure, the rats were conscious and breathing spontaneously.

2.4. Treatment with vehicle or MP

In **Part 1**, 156 rats were randomly divided into six groups, as follows: control (C) group (ambient air exposure and no treatment, n = 6); S group (30-min smoke inhalation, n = 30); S + NS group (30-min smoke inhalation and treated with 1 mL/kg NS intraperitoneally, n = 30); and S + 40 mg/kg MP, S + 4 mg/kg MP, and S + 0.4 mg/kg MP groups (30-min smoke inhalation and 40, 4, or 0.4 mg/kg MP (diluted in NS), respectively, intraperitoneally, n = 30 in each group). MP or NS was administered at 60 min before smoke inhalation. All smoke exposure experiments were carried out at the same time at 8:00 am. Twenty-four hour after smoke inhalation, survival rates were calculated and six rats were randomly selected from each group and sacrificed by

pentobarbital sodium overdose (100 mg/kg body weight), and blood, lung tissue, and bronchoalveolar lavage fluid (BALF) were collected immediately.

In **Part 2**, 178 rats were randomly divided into five groups: C group (ambient air exposure and no treatment, n = 18); S group (n = 40); S + MP(1D); S + MP(3D); and S + MP(7D) (30-min smoke inhalation and rats in each treatment group were administered 4 mg/kg MP (diluted in NS) intraperitoneally for 1, 3, or 7 consecutive days, respectively; n = 40 in each group). MP was intraperitoneally administered 1 h before smoke inhalation. Survival rates were calculated up to 28 days after exposure. In all of the above groups, six rats were randomly selected and sacrificed by an overdose of pentobarbital sodium (100 mg/body weight). Lung and blood tissues were collected at 7, 14, and 21 days after smoke inhalation.

2.5. Blood analysis

At each time point, about 1 mL of arterial blood was obtained from the left carotid artery. Arterial partial pressure of oxygen (PaO_2), arterial partial pressure of carbon dioxide ($PaCO_2$), and pH were measured using a blood gas analyzer (Premier 3000, Instrumentation Laboratory, Boston, MA, USA).

2.6. Bronchoalveolar lavage procedure

Bronchoalveolar lavage (BAL) was performed by perfusing the left lung via a 20-gauge tracheostomy using an angiocath with cold PBS containing 0.5 mM EDTA in 1 mL aliquots, and gently removing the fluid to collect an approximate total volume of 2.75 mL (three injections were made in total). The fluid was immediately placed on ice. Approximately 70% of the fluid in the first injection and 90% of the fluid in subsequent injections was recovered.

2.7. Bronchoalveolar lavage analysis

To determine the abundance of neutrophils and macrophages in the BALF, which reflects the relative abundance of these cell types in the alveolar space, BAL was performed as described above. The total amount of cells from 100 μL BALF was counted using a hemocytometer. Then, the BALF was centrifuged at 3000 ×*g* for 5 min, and cell pellets were re-suspended in 1 mL of Hank's balanced salt solution (HBSS) containing 0.5% BSA. Differential cell assays were conducted using Giemsa staining, and 400 cells per slide were counted in randomly selected high-powered fields (×1000). For BAL protein analysis, the BALF supernatant was collected after centrifugation and stored at −80 °C before protein and cytokine assay. The BAL protein concentration was measured using the Bio-Rad Protein Assay kit. TNF-α, IL-6, IL-17a, ATIII, TATc, and PAI-1 levels were measured using ELISA.

2.8. Lung histology

The upper lobe of the right lung was perfused with 300 μL of paraformaldehyde. The trachea was then ligated using a 5–0 suture to keep the lungs inflated. The tissue was placed in formalin and fixed for 48–72 h before being processed and embedded in paraffin. Hematoxylin and eosin (H&E)-stained lung samples from all groups were investigated to assess the degree of injury, which was calculated using the lung injury scoring system of the American Thoracic Society [35] (Table 1). At least 20 random regions were scored independently from 0 to 2 at a magnification of ×400 in a blinded fashion. The final lung injury score for each lung was calculated as follows: score = [(20 × A) + (14 × B) + (7 × C) + (7 × D) + (2 × E)]/(number of fields × 10).

Masson's trichrome and Sirius red staining of the right upper lobe were used to assess the degree of fibrosis in all groups. Histopathologic analysis was performed by two experienced veterinary pathologists (General Navy Hospital), who were blinded to the experimental conditions. For the qualitative assessment of pulmonary fibrosis, the collagen tissue area was measured after Sirius red staining using an imaging analysis program, Image pro-Plus 6.0 (Media-Cybernetics, Silver Spring, MD). Briefly, ten random, high-power field images of the lungs were acquired using a digital camera (DP20, Olympus, Tokyo, Japan) on a light microscope (BX51, Olympus, Japan) and colors that were not of interest were removed via the replace mode. The adapted images were converted to greyscale and the area of expression was then located by adjusting the threshold. The percent area of expression was represented by the positive pixels on the labelled areas, whereas collagen deposition was measured using the septal area and image. The intensity score was used to qualify the percent area of collagen (5 grades: 1, no staining (< 10%); 2, mild staining (10%–30%); 3, moderate staining (30%–60%); 4, strong staining (60%–80%); and 5, severe staining (80%–100%)) [36].

Table 1
Lung injury scoring system of the American Thoracic Society.

	Score per field		
Parameter	0	1	2
Neutrophils in the alveolar space	0	1–5	> 5
Neutrophils in the interstitial space	0	1–5	> 5
Hyaline members	0	1	> 1
Proteinaceous debris filling the airspaces	0	1	> 1
Alveolar septal thickening	< 2 fold	2–4 fold	> 4 fold

2.9. Wet-to-dry weight ratio of lung tissues

The right accessory lobe of the lung was dissected and washed in HBSS. Excess HBSS was wiped away with ultra-soft tissues, and the lung was placed on a piece of aluminum foil and weighed immediately (wet weight). The lungs were dried in an oven at 60 °C and weighed again 48 h later (dry weight). The wet-to-dry ratio (W/D) is a commonly used marker for edema formation.

2.10. Western blot analyses

Western blots were performed using antibodies against MPO, HMGB1, IL-10, and CTGF. Protein (20 μg) from each sample was loaded into an SDS-polyacrylamide gel. The separated proteins were transferred to a polyvinylidene fluoride membrane, which was blocked with 5% skim milk, followed by incubation with a 1:1000 dilution of the indicated antibody. Antibody binding was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies. Bands were visualized using ECL and Kodak GBX developer/fixer. The blots were exposed to X-ray film (Fujifilm Medical, Stamford, CT, USA). For quantitation, densitometric analysis of the immunoblot was performed using an Alpha Imager 2200 digital imaging system (Digital Imaging System, San Leandro, CA, USA).

2.11. RNA extraction and quantitative reverse transcription (qRT)-PCR

The RNeasy Micro Kit (Thermo Scientific) was used to extract RNA from lung tissue. Reverse transcription was performed on RNA using the iScript™ Select cDNA synthesis Kit (BioRad, Hercules, CA, USA). RNA quality was assessed using an Agilent 2100 bioanalyzer (Santa Clara, CA, USA). RT-PCR was performed on cDNA using the UltraSYBR Mixture kit (Cwbiotech, Beijing, China) with a 0.2 μM concentration of forward and reverse primers. Primers were custom synthesized by Sunnybio (Shanghai, China). The fold-change was calculated using two-step normalization of expression against corresponding GAPDH, and using the first and corresponding time-matched control samples as the second normalization reference ($2^{\Delta(\Delta CT)}$). The purity of the product was

Table 2
List of qRT-PCR primers used to determine expression levels in different groups.

Gene	Forward/Tm	Reverse/Tm
α-SMA	GTC CCA GAC ATC AGG GAG TAA	TCG GAT ACT TCA GCG TCA GGA
TGF-β1	GACTCTCCACCTGCAAGACCAT	GGG ACT GGC GAG CCT TAG TT
GAPDH	TGGCCTTCCGTGTTCCTAC	GAGTTGCTGTTGAAGTCGCA

α-SMA, α smooth muscle actin; TGF-β1, transforming growth factor-β1; GAPDH, glyceraldehyde phosphate dehydrogenase.

assessed using melting-curve analysis. The primer sequences used to analyze the expression levels of different markers are shown in Table 2.

2.12. Statistical analysis

Values are presented as the mean ± standard error of the mean (SEM). Differences between groups were compared using a two-tailed, unpaired *t*-test or a one-way ANOVA, followed by Dunnett's multi-comparison test using Prism GraphPad 6 (GraphPad, La Jolla, USA). Differences were considered significant when p was < 0.05. All assays were performed at least in duplicate. For survival analysis, the Kaplan–Meier method was used followed by a log-rank test.

3. Results

3.1. MP significantly improves the survival rate after acute smoke inhalation

The significance of the protective effects of MP compared with those of the smoke group was assessed using a log-rank test. Animals treated with 40, 4, or 0.4 mg/kg MP exhibited higher survival rates (66%, 85%, and 60%, respectively) compared with the smoke group (31%) and the S + NS group (37%) ($p < 0.05$, S + 4 mg/kg and S + 40 mg/kg groups versus S, S + NS and S + 0.4 mg/kg groups, separately). The medium MP dose (4 mg/kg) yielded a higher protective effect compared with the high and low MP doses ($p < 0.05$, S + 4 mg/kg group versus S + 40 mg/kg group) (Fig. 1).

3.2. Effects of different MP doses on alleviating acute lung injury

Histopathological analysis showed the massive infiltration of neutrophils into the intra-alveolar septa and alveolar spaces in the S group

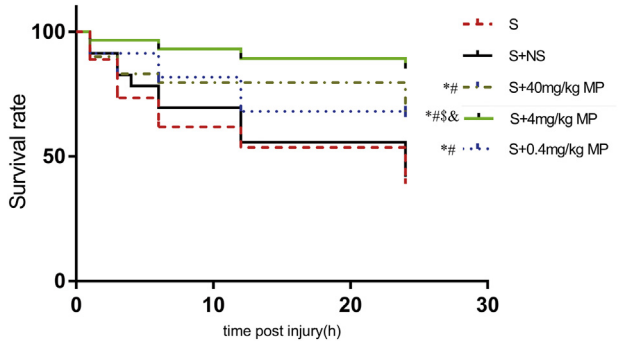

Fig. 1. Effect of different dosages of MP treatment on survival rates in rats with smoke inhalation. Percentage of 24-h survival in rats subjected to smoke inhalation or NS or different MP doses 1 h before smoke inhalation. S, n = 30; S + NS, n = 30; S + 40 mg/kg MP, n = 30; S + 4 mg/kg MP, n = 30; and S + 0.4 mg/kg MP, n = 30. The survival rate was compared using the Kaplan–Meier method and the log-rank test. $^{*}p < 0.05$, compared with the S group; $^{\#}p < 0.05$, compared with the S + NS group; $^{\$}p < 0.05$, compared with the S + 40 mg/kg MP group; $^{\&}p < 0.05$, compared with the S + 0.4 mg/kg MP group.

(Fig. 2B). Alveolar hemorrhage, edema of alveolar septa, and proteinaceous debris in the alveolar space were also observed in the S group. These changes were also found in the S + NS group (Fig. 2C). However, these inflammatory changes were strikingly attenuated in rats treated with S + 40 mg/kg or 4 mg/kg. Further evaluation of these inflammatory changes showed that lung injury scores were significantly lower in rats treated with 4 mg/kg or 40 mg/kg compared with the S + NS and 0.4 mg/kg groups ($p < 0.05$, S + 4 mg/kg vs S + 0.4 mg/kg and S + NS; S + 40 mg/kg vs S + 0.4 mg/kg and S + NS) (Fig. 2G). The protein level in BALF, which reflects the degree of lung edema and damage to the alveolar–capillary barrier 24 h after smoke challenge (Fig. 2H), showed that 4 mg/kg and 40 mg/kg doses of MP were associated with significantly reduced protein concentrations compared with in the S + NS groups. Additionally, 4 mg/kg MP markedly reduced the protein level and lung injury score compared with the 40 mg/kg group ($p < 0.05$, S + 4 mg/kg vs S + 40 mg/kg).

The W/D weight ratio was used as an index of water accumulation in the lung, which is an indicator of lung edema. At 24 h after smoke exposure, the lung W/D ratio in the S and S + NS groups was significantly elevated compared with the control group, and this ratio was significantly decreased in all MP-treated groups. There was no significant difference in the W/D ratio between the S + 40 mg/kg and S + 4 mg/kg groups ($p > 0.05$, S + 4 mg/kg vs S + 40 mg/kg), whereas it was significantly lower in the S + 4 mg/kg group compared with the 0.4 mg/kg group ($p < 0.05$, S + 4 mg/kg vs S + 0.4 mg/kg) (Fig. 2I).

The number of inflammatory cells in the S and S + NS groups was dramatically increased at 24 h after smoke challenge, and over 55% of these cells were identified as neutrophils. No significant differences were found between these two groups. Notably, rats treated with 4 or 40 mg/kg of MP had a significantly lower number of inflammatory cells compared with S + NS-treated rats at 24 h ($p < 0.05$, S + 4 mg/kg vs S + 0.4 mg/kg and S + NS; S + 40 mg/kg vs S + 0.4 mg/kg and S + NS) (Fig. 2J, L). However, treatment with 0.4 mg/kg MP did not reduce the number of inflammatory cells and neutrophils at any time point after smoke challenge. Conversely, treatment with smoke and smoke + NS significantly reduced the numbers of macrophages in the lungs at 24 h, and 4 or 40 mg/kg MP treatment resulted in a significantly higher percentage of macrophages in the alveolar space at 24 h after smoke challenge ($p < 0.05$, S + 4 mg/kg vs S + 0.4 mg/kg and S + NS; S + 40 mg/kg vs S + NS) (Fig. 2K).

Results of arterial blood gas analysis are shown in Fig. 1. There was a significant increase in $PaCO_2$ and a significant decrease in pH and PaO_2 after smoke inhalation, and no differences were found between the S and S + NS groups. Treatment with 4 or 40 mg/kg MP significantly improved the decrease in pulmonary gas exchange efficiency while 0.4 mg/kg of MP only slightly improved PaO_2. However, 4 mg/kg MP treatment greatly improved PaO_2 compared with 0.4 mg/kg MP ($p < 0.05$, S + 4 mg/kg vs S + 0.4 mg/kg) (Fig. 2M, N, O).

3.3. Effect of different MP doses on attenuating acute-phase local and systemic inflammation

IL-6, MPO, IL-17a, IL-10, HMGB1, and TNF-α are cytokines critical for the development of ALI. TNF-α, IL-17a, and IL-6 concentrations were significantly increased at 24 h after smoke administration. MP treatment (4 or 40 mg/kg) significantly decreased IL-6, MPO, IL-17a, and TNF-α levels in the BALF and plasma 24 h after smoke exposure ($p < 0.05$, S + 4 mg/kg vs S + NS and S + 0.4 mg/kg; S + 40 mg/kg vs S + NS and S + 0.4 mg/kg). However, there was no significant effect on inflammatory cytokine production when 0.4 mg/kg of MP was used, except for IL-17a. No differences in cytokine levels were found between 4 and 40 mg/kg MP ($p > 0.05$, S + 4 mg/kg vs S + 40 mg/kg) (Fig. 3A–F).

Western blot results showed that the production of HMGB1 and MPO were significantly increased at 24 h after smoke administration.

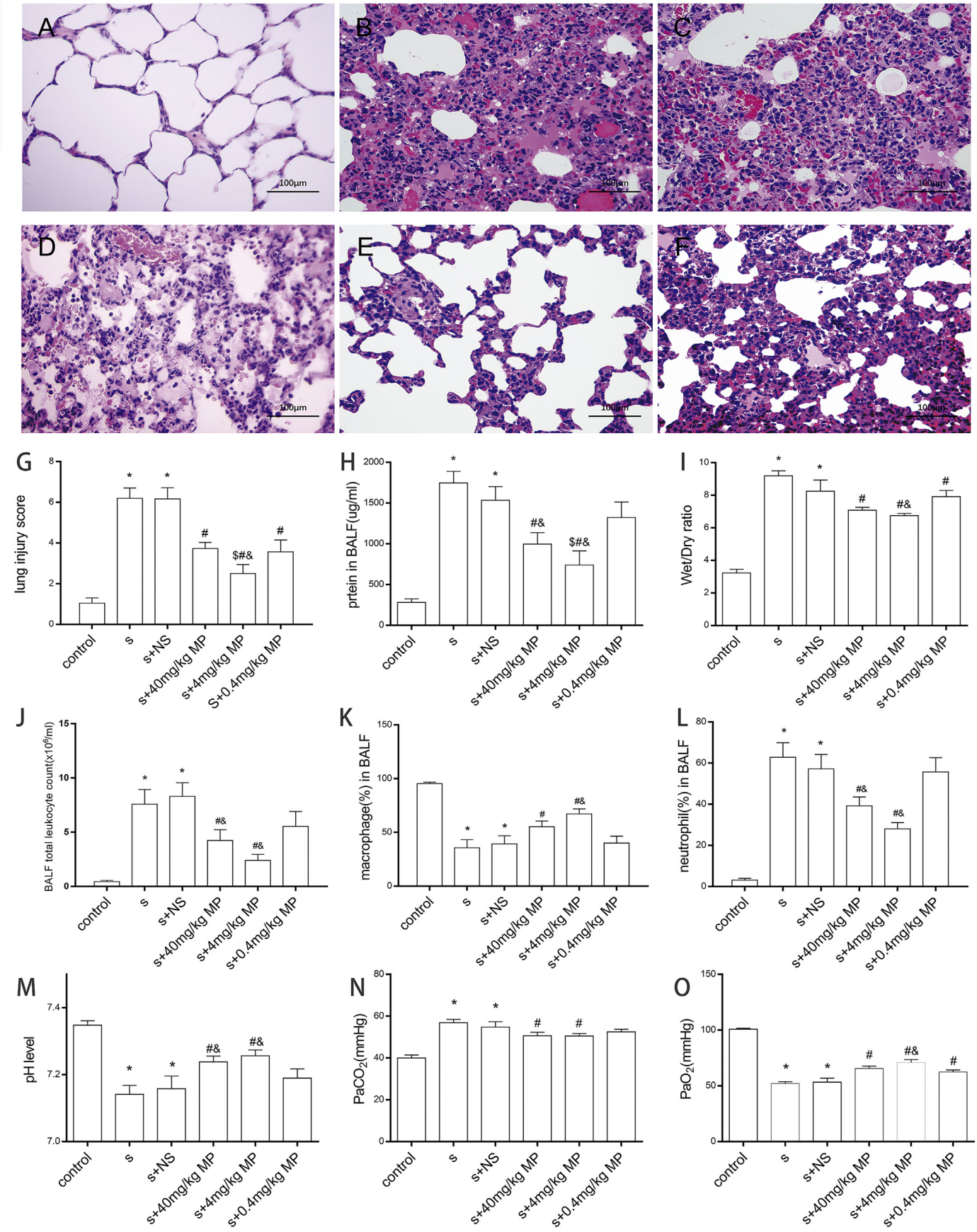

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Fig. 2. Effect of different dosages of MP treatment on lung histology, leukocytes in BALF, and blood gas alterations in rats with smoke inhalation. At 1 h before smoke inhalation, rats were treated with NS, or 40, 4, or 0.4 mg/kg MP. Lung sections, prepared 48 h after smoke administration, were stained with hematoxylin and eosin. Untreated rats were used as controls. Representative images of control (A), smoke (B), smoke + normal saline (NS) (C), smoke + 40 mg/kg (D), smoke + 4 mg/kg (E), and smoke + 0.4 mg/kg MP (F)-treated rats are shown at × 400 magnification. (G) The lung injury scores calculated for lung sections from each group of treated rats were evaluated as previously described [31]. The data are shown as the mean and SEM (n = 6). (H) The BALF protein level was examined in each treatment group (n = 6) 24 h after smoke challenge. (I) The lung tissues used to calculate the W/D ratio were collected immediately after 24 h of smoke inhalation (n = 6). The total number of inflammatory cells was determined using a hemocytometer (J), while the proportions of macrophages (K) and neutrophils (L) were evaluated post cytospin. Arterial blood samples were obtained and analyzed using a spectrophotometer (ABL 520; Radiometer; Copenhagen, Denmark) to detect the partial pressure of oxygen (PaO_2) (M), partial pressure of carbon dioxide ($PaCO_2$) (N), and pH (O) at 24 h after smoke administration (n = 6). Each column represents the mean and SEM. $^{*}p < 0.05$, compared with the control group; $^{\#}p < 0.05$, compared with the S + NS group; $^{\$}p < 0.05$, compared with the S + 40 mg/kg MP group; $^{\&}p < 0.05$, compared with the S + 0.4 mg/kg MP group.

All MP treatment groups suppressed this increase to different degrees, but the 4 mg/kg MP group showed the most potent effect. IL-10 is produced by macrophages and lymphocytes and it inhibits the development of lung injury in severe ARDS rats by suppressing the activation of immune and inflammatory responses. Levels of IL-10 in the lungs of rats in each group were significantly increased after 4 and 40 mg/kg MP ($p < 0.05$, S + 4 mg/kg vs S + NS and S + 0.4 mg/kg; S + 40 mg/kg vs S + NS and S + 0.4 mg/kg). No significant differences were observed between these two groups, but HMGB1 and MPO levels were markedly decreased ($p < 0.05$, S + 4 mg/kg vs S + NS; S + 40 mg/kg vs S + NS) (Fig. 3G–J).

3.4. Impact of MP on changes in local coagulation

We evaluated changes in coagulation in the BALF by ELISA using three parameters: TATc, ATIII, and PAI-1, which are procoagulatory, anticoagulatory, and fibrinolytic biomarkers, respectively. We found profound coagulation activation after smoke inhalation, with an increase in TATc and a decrease in ATIII. Fibrinolysis was impaired, with an increase in PAI-1. MP (4 and 40 mg/kg) markedly improved coagulation conditions by decreasing TATc (Fig. 4A) and PAI-1 (Fig. 4C), and increasing ATIII (Fig. 4B). No differences in these parameters were found between 4 and 40 mg/kg MP ($p > 0.05$, S + 4 mg/kg vs S + 40 mg/kg). Treatment with 0.4 mg/kg MP did not contribute to this profile.

3.5. The impact of different MP treatment durations on the survival rate in rats after 28 days

Smoke inhalation caused severe ALI, leading to a mortality rate of 50% within 72 h, and this mortality rate did not change significantly on subsequent days. Rats in this study were treated with 4 mg/kg MP for 1, 3, or 7 days. Compared with the S group, rats with all three doses of MP had an improved survival rate ($p < 0.05$, S vs all three MP groups), and there was no significant difference among these three groups ($p > 0.05$, among all three MP groups) (Fig. 5).

3.6. The impact of a medium MP dose on pulmonary fibrosis

Smoke inhalation gradually leads to fibrosis. Masson's Trichrome and Sirius red staining at 7, 14, and 28 days was quantified in lung tissue to measure lung fibrin and collagen deposition. Direct staining of collagen in lung slices using Masson's Trichrome stain revealed a gradual increase in collagen deposition in injured rats at 7, 14, and 28 days compared with uninjured rats. Sirius red staining represented collagen deposition, which was attenuated after 3 and 7 days of MP treatment ($p < 0.05$, S vs MP(3D) and MP(7D)). This attenuation was reflected by the mean opacity density of Sirius red staining. One day of MP treatment showed no benefit regarding fibrotic improvement ($p > 0.05$, S vs MP(1D)) (Fig. 6A–D).

TGF-β1 and α-SMA are potential fibrotic mediators, and they were also detected within lung tissues. qRT-PCR showed a strong increase in TGF-β1 and α-SMA expression in the lung in a time dependent manner.

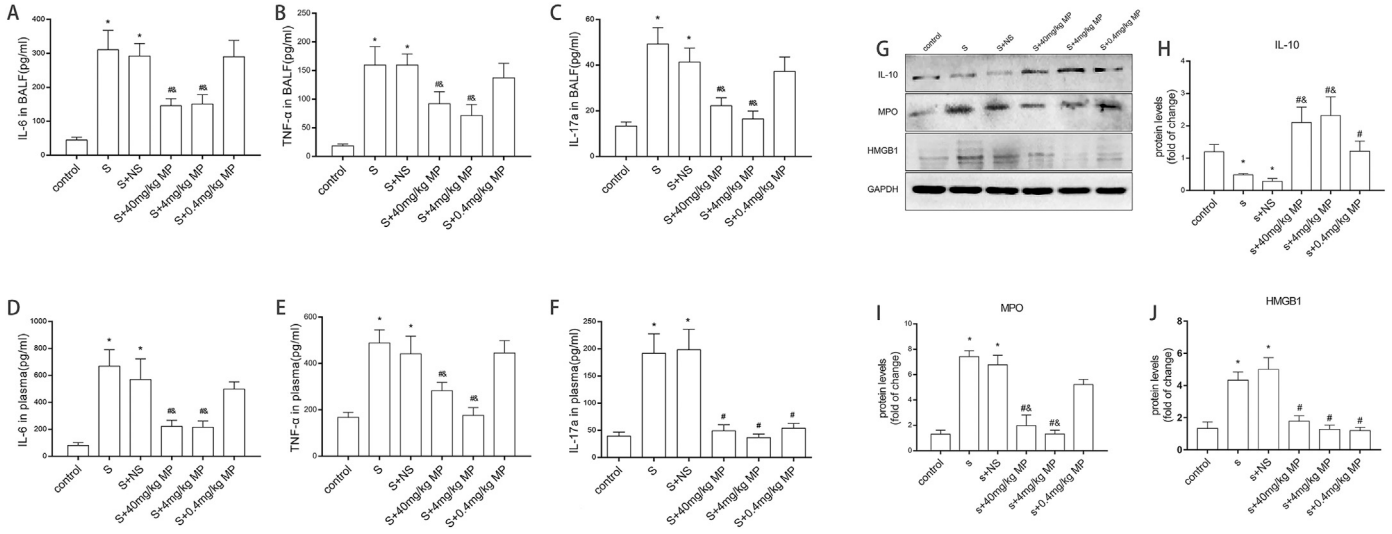

Fig. 3. MP decreases proinflammatory cytokine levels in the BALF and plasma and increases the level of IL-10 in the lung tissue of rats with smoke inhalation. At 1 h before smoke inhalation, rats (n = 6 in each group) were treated with NS, or 40, 4, or 0.4 mg/kg of MP. The S group received no treatment before smoke inhalation. Naive rats were used as controls. Plasma and BALF samples were taken at 24 h after smoke administration. After BAL, the right lower lung was harvested from rats at 24 h after smoke inhalation. TNF-α, IL-6, and IL-17a were measured in the BALF and plasma by ELISA (A–F) and the concentrations of HMGB1, MPO, and IL-10 were measured in lung tissue by Western blot (G–J). Each column represents the mean and SEM. $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the S + NS group, $^{\&}p < 0.05$ compared with the S + 0.4 mg/kg MP group.

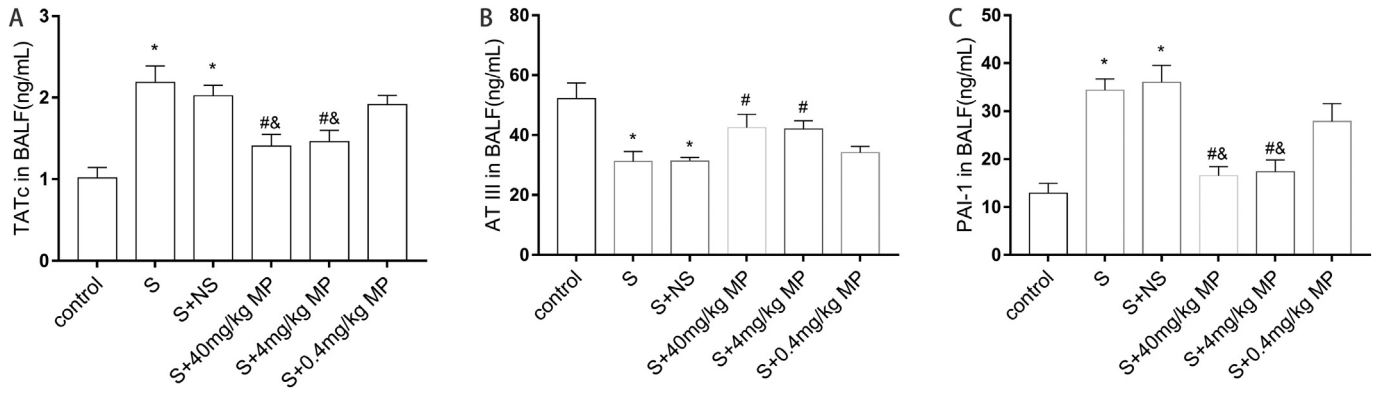

Fig. 4. Treatment with 4 or 40 mg/kg MP improves coagulatory and fibrinolytic disorders in the BALF. At 1 h before smoke inhalation, rats (n = 6 in each group) were treated with NS, or 40, 4, or 0.4 mg/kg MP. The S group received no treatment before smoke inhalation. Naive rats were used as controls. BAL was performed at 24 h after smoke administration. The TATc, ATIII, and PAI-1 concentrations in BALF were measured by ELISA. Each column represents the mean and SEM. $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the S + NS group, $^{\&}p < 0.05$ compared with the S + 0.4 mg/kg MP group.

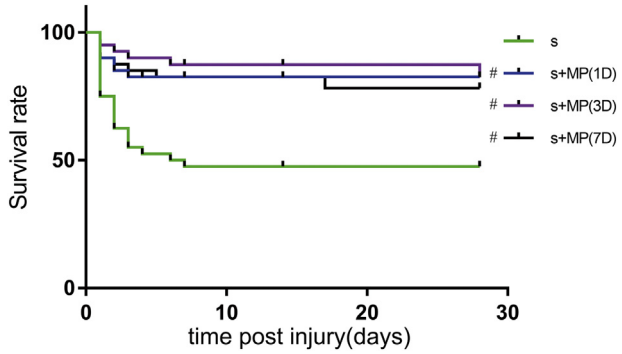

Fig. 5. Effect of different durations of MP treatment on survival rates in rats with smoke inhalation. The percentage of rats surviving at 28 days after smoke administration or different durations of MP treatment is shown. Rats were treated with MP for 1, 3, or 7 days. S, n = 30; S + MP(1D), n = 30; S + MP(3D), n = 30; and S + MP(7D), n = 30. The survival rate was compared using the Kaplan–Meier method and the log-rank test. $^{\#}p < 0.05$ vs S group.

All MP treatments significantly attenuated the TGF-β1 increase ($p < 0.05$, S vs all MP groups), while only 3- and 7-day treatments decreased α-SMA mRNA levels 28 days after smoke exposure ($p < 0.05$, S vs MP(3D) and MP(7D)) (Fig. 6E, F). Both 3- and 7-day treatments were more efficient compared with the 1-day treatment. Western blot was used to evaluate CTGF protein levels in lung tissues (Fig. 6G). CTGF is a matrix and cellular protein that plays a key role in tissue development and remodeling, interacting with a variety of growth factors such as transforming growth factor (TGF)-β. We found that CTGF was markedly increased in the S group 28 days after smoke inhalation and was significantly decreased after 1, 3, and 7 days of MP treatment. There was no significant difference in CTGF levels among the three MP groups ($p > 0.05$, among all three MP groups).

3.7. Effects of different MP treatment durations on inflammatory cytokines in the fibrotic phase

ELISA was used to measure IL-6, IL-17a, and TNF-α levels in the BALF and plasma. We found that levels of three cytokines, but not IL-17a in plasma, were still high in the BALF and plasma in the smoke group 28 days after smoke inhalation (Fig. 7A–F). However, all MP treatments had no difference in plasma cytokine levels compared with the smoke group, expect for TNF-α in the BALF after 3 days MP treatment ($p < 0.05$, S vs MP(3D)) (Fig. 7B) and IL-17a in the BALF after 1 day of MP treatment ($p < 0.05$, S vs MP(1D)) (Fig. 7C). Western blot results showed that HMGB1 and MPO production was significantly increased after 28 days of smoke administration. All MP treatment groups showed suppression of this increase to different degrees, but there was no significant difference among all the MP groups ($p > 0.05$, among all three MP groups) (Fig. 7G, I, J). No significant differences in IL-10 were found among all groups (Fig. 7H).

3.8. Local coagulation states 28 days after smoke inhalation

We measured TATc, ATIII, and PAI-1 levels in the BALF in all groups 28 days after smoke inhalation. We found there was still a high level of TATc in the BALF from the smoke group, which was markedly attenuated after 3 and 7 days of MP treatment ($p < 0.05$, S vs MP(3D) and MP(7D)), while a single dose of MP had no effect on TATc levels. Although there was a trend toward a decrease in ATIII and an increase in PAI-1, no significant difference was found between the control and smoke groups ($p > 0.05$, among all groups). Each MP-treated group showed no significant difference compared with the smoke group (Fig. 8).

4. Discussion

Smoke inhalation induces ALI, although this rarely occurs, and it contributes greatly to mortality in fire disasters. The underlying mechanisms in this type of trauma are complex and cross-linked, and lead to severe complications in the early and late phases. With the development fire-proof materials in household supplies, inhalation injury has become one of the most lethal complications in burn patients [4]. We developed a smoke inhalation injury model using composite materials to mimic real world conditions and explore the optimal MP strategy for SI-ALI management. We showed that: (1) a single, medium dose of MP (4 mg/kg) significantly improved the survival rate of SI-ALI rats compared with low-dose (0.4 mg/kg) and high-dose (40 mg/kg) MP; (2) MP therapy attenuated acute-phase injury by reducing leakage of the alveolar–capillary barrier, attenuating local coagulation disturbances, and inhibiting local and systemic inflammatory cytokine production; and (3) MP treatment ameliorated fibrotic changes in the late phase of SI-ALI when administered early and sequentially for 3 or 7 days, but prolonged treatment (7 days) was not associated with a better outcome compared with the medium-term strategy (3 days).

Although GCs administered in the early phase of lung inflammation appear to resolve ALI and ARDS because of their potent anti-inflammatory effect [37], GC application clinically remains controversial. The main reason for this controversy is the discrepancy between the doses used in clinical trials [17,22,38,39]. Because SI-ALI is caused by bursting fire disaster and rarely happens, systematic and high-quality clinical trials, which minimize confounding factors such as

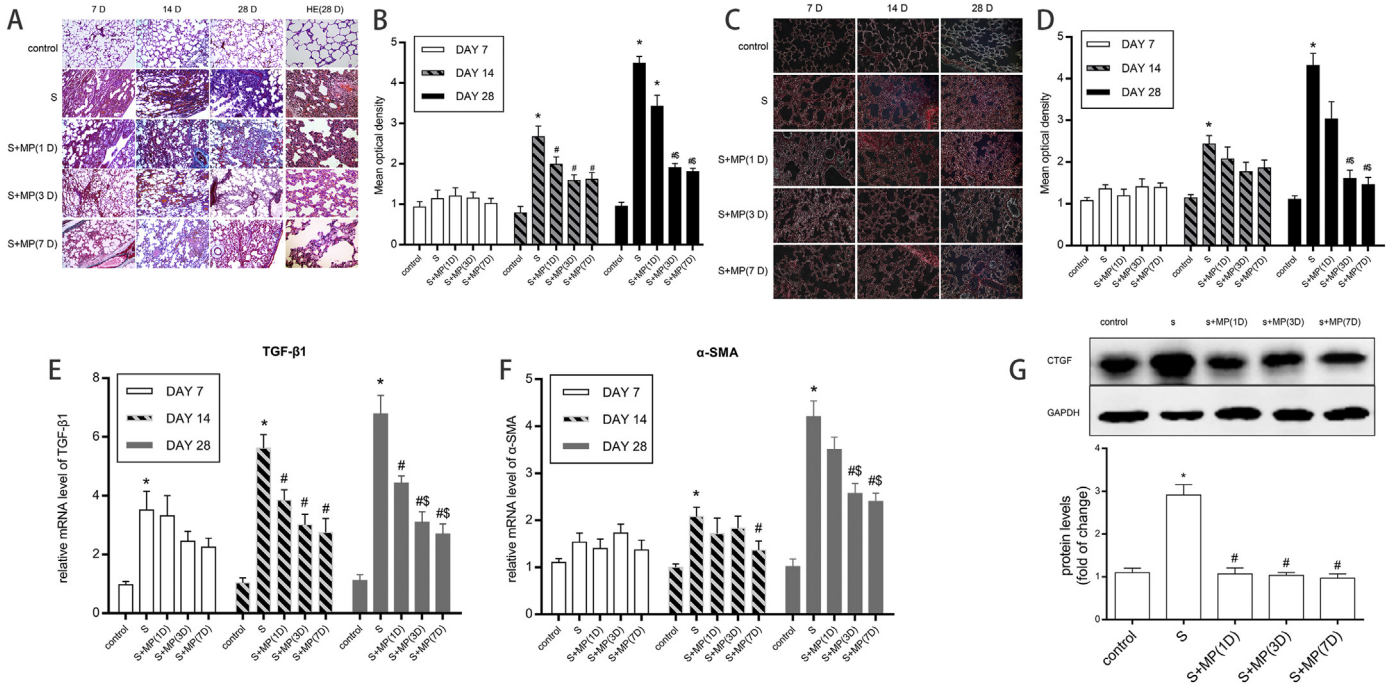

Fig. 6. Effect of different durations of MP treatment on fibrotic changes in lung tissues on days 7, 14, and 28 after smoke inhalation. Rats were treated with smoke for 30 min and MP for 0, 1, 3, and 7 days. Lung sections were collected at 7, 14, and 28 days after smoke inhalation and were stained with Mallory's Trichrome Masson (A and B; collagen in blue for 7D, 14D, 28D), hematoxylin-eosin (A; H&E 28D), or Sirius red (C and D; collagen appears as bright birefringent deposits under epipolarized light). Relative TGF-β (E) and α-SMA (F) mRNA expressions were examined using qRT-PCR and the results were normalized to GAPDH mRNA expression. CTGF expression was measured in lung tissues using Western blotting (G). Untreated rats were used as controls. Images are shown at ×200 magnification. Mallory's Trichrome Masson and Sirius red staining was measured qualitatively by selecting the positive stained area, and calculating and graphing the mean ratio to each microscopic field (×200) (n = 6, 10–15 images per rat were captured for quantification). Each column represents the mean and SEM. $^{*}p < 0.05$, compared with the control group; $^{\#}p < 0.05$, compared with the S group; $^{\$}p < 0.05$, compared with the S + MP(1D) group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

heterogeneous severity scores, sample size, and comorbidities, are hard to conduct, resulting in controversy regarding whether GC should be adopted in SI-ALI [4,40]. In burn centers, GCs are used with caution because they can increase the risk of infection of patients with burn injury [3,41]. Therefore, we performed this preclinical experiment to explore the efficiency of different MP dosages and durations for isolated smoke inhalation injury. Direct thermal damage is generally confined to the supra-glottic airway and is uncommon below the vocal cords because of the highly efficient heat exchange system in the oropharynx and nasopharynx. Furthermore, the low heat capacity of the airways and the reflex mechanism of laryngeal closure lead to edema of the supraglottic structures that obstructs the upper airway just hours after

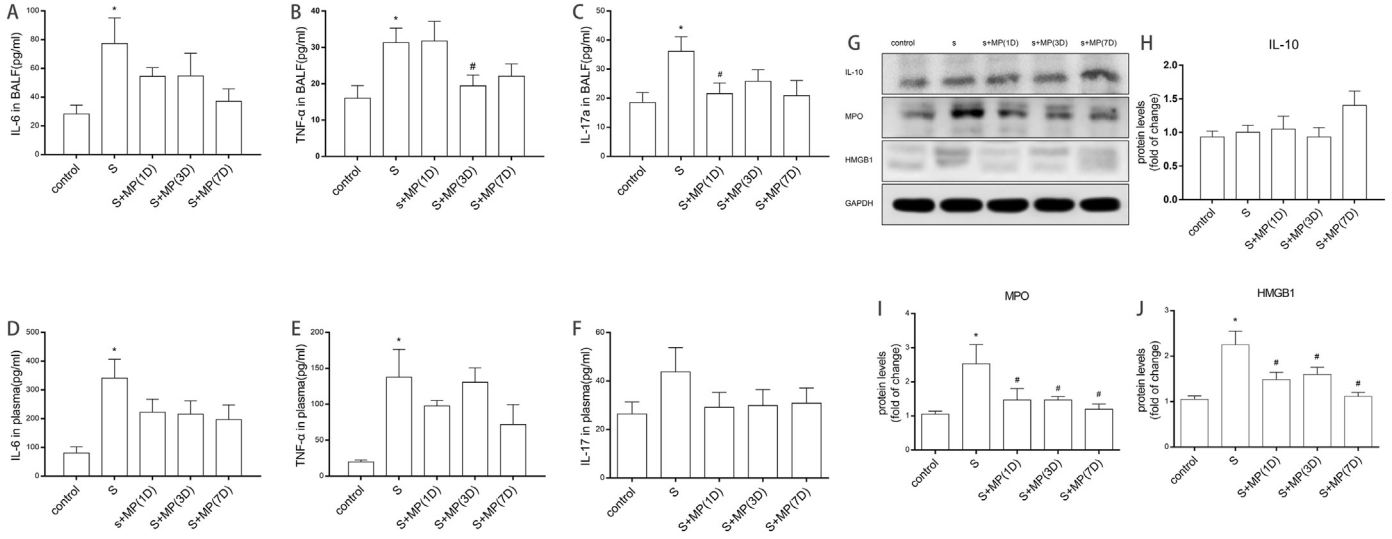

Fig. 7. Effect of different durations of MP treatment on proinflammatory cytokine levels in the BALF and plasma and protein expressions in lung tissues 28 days after smoke inhalation. Rats were treated with smoke for 30 min and MP for 0, 1, 3, and 7 days. Lung tissues and plasma samples were collected and BAL was performed 28 days after smoke inhalation. IL-6, IL-17a, and TNF-α concentrations in the plasma and BALF were tested using an ELISA (A–F) and HMGB1, MPO, and IL-10 expressions were measured in lung tissues using Western blotting (G–J). Untreated rats were used as controls. Each column represents the mean and SEM (n = 6 in each group). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the S group.

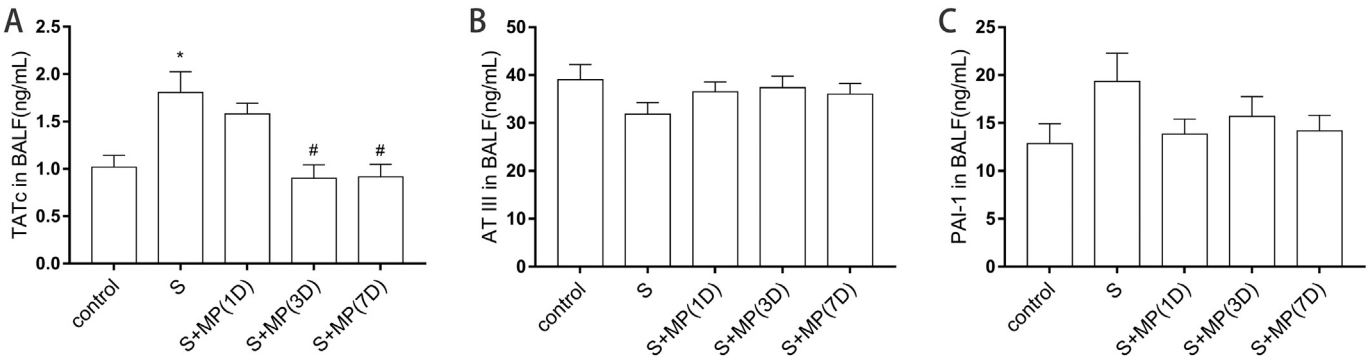

Fig. 8. Effect of different durations of MP treatment on coagulation and fibrinolysis 28 days after smoke inhalation. Rats were treated with smoke for 30 min and MP for 0, 1, 3, and 7 days. Lung tissue samples were collected and BAL was performed 28 days after smoke inhalation. Concentrations of TATc (A), ATIII (B), and PAI-1 (C) in the BALF were measured using ELISA. Untreated rats were used as controls. Each column represents the mean and SEM (n = 6 in each group). $^{*}p < 0.05$ compared with the control group, $^{\#}p < 0.05$ compared with the S group at each point.

insult, which progresses quickly with burn edema during resuscitation, contributing to a considerable proportion of deaths during fire disasters [42]. Consequently, we excluded thermal injury to the upper glottis by controlling the temperature of smoke in the chamber and burn injury by reducing flame exposure of the rats. As there is limited evidence for the management of supraglottic airway damage caused by thermal injury in fire disasters [31], whether GC could alleviate this type of injury needs further independent and specified study.

In the first part of our study, we tested three different MP doses for acute-phase management. We found that a medium dose of MP significantly improved the survival rate compared with low-dose and high-dose groups. Cytokines in the BALF and plasma had a similar effect to the high- or medium-dose by attenuating the release of inflammation factors. These changes led to an improvement in blood gas levels in the high- and medium-dose groups. IL-10 acts as an anti-inflammatory cytokine that inhibits proinflammatory cytokines [43], and the increase in IL-10 levels indicated that MP attenuated smoke inhalation-induced proinflammatory cytokine levels. These results suggest that MP improves the survival rate and reduces the severity of lung injury by inhibiting proinflammatory cytokines and promoting anti-inflammatory cytokines.

Activation of coagulation during inflammation is a physiologic response that helps to contain inflammation. Although coagulation is a precondition for secondary reparative processes, it may also increase inflammation if uncontrolled. Many studies suggested that pulmonary coagulation disturbances and fibrin deposition in the alveolar compartment are attributed to inhalation injury [3,9,44,45].

A study showed that blocking coagulation or stimulating fibrinolysis could theoretically reduce the inflammatory response, thereby reducing lung injury [46]. In our study, marked procoagulant and antifibrinolytic changes were found in the BALF after smoke inhalation. This is consistent with the results of previous studies, which reported increased procoagulant activity in the alveolar space in patients with ALI/ARDS [9,47]. We found that in the acute phase, treatment with 4 or 40 mg/kg MP was associated with improved coagulation and fibrinolysis. The ATIII level in BALF was also markedly increased after MP treatment, similar to that of TATc. The improvement in coagulation and fibrinolysis states may contribute to a reduction in lung injury score.

The high dose of MP did not exert more of an effect on lung injury improvement compared with the medium dose of MP, and the explanation for this is complex. A substantial portion of the genome is targeted by the GR, with some studies indicating that up to 20% of expressed genes were positively or negatively regulated by GCs [48]. A growing body of evidence indicates that the sensitivity and specificity of GC signaling results from the molecular heterogeneity of GR proteins [49] and a high dose of dexamethasone impaired GR mRNA expression [50], which may reduce the effectiveness of GC for positive gene modulation. A study by Liu et al. showed that high-dose GC treatments aggravated, rather than reversed, phosgene pulmonary toxicity [51]. The above results may explain why the high dose of GC had no greater effect than the medium dose in ALI treatment.

In this study, we found that high doses of MP (40 mg/kg) had no additional beneficial effect on the survival rate in our ARDS model and that the administration of low-dose MP (0.4 mg/kg) did not suppress cytokine production in the lungs of rats in the S group. Thus, we used a medium dose (4 mg/kg) to study the fibrotic phase.

Early complications of inhalation injury have been well described in the literature. However, there is a paucity of studies on the long-term or delayed complications from inhalation injury [47,52]. A model established by Zhu et al. reported that collagen deposits were observed 28 days after smoke injury [11]. However, few studies have explored treatment in the late phase of SI-ALI, because the survivors of ARDS usually go through exudative, proliferative, and fibrotic phases. The final, or fibrotic, phase of ARDS does not occur in all patients but it has been linked to prolonged mechanical ventilation and higher mortality rates [12]. We extended our study to the fibrotic phase after SI-ALI.

Masson and Sirius staining showed a gradual increase in fibrin and collagen deposition from 7 to 28 days after smoke inhalation, which were similar to results obtained using chlorine, sulfur dioxide, or bleomycin-induced lung inflammation and fibrosis models [53–55]. In mammals, TGF-β1 is considered a principal profibrotic agent that is upregulated in areas of regeneration and remodeling foci in lung fibrosis. Some of its activities include promoting collagen synthesis promotion and deposition, producing extracellular matrix, inducing the differentiation of fibroblasts to myofibroblasts, and inhibiting fibroblast autophagy [56–58]. We found that after MP administration for 3 or 7 days, fibrotic phase progression was markedly attenuated, which was reflected by TGF-β1 and α-SMA mRNA levels. These changes suggested that the antifibrotic effects could be partly explained by the suppression of TGF-β1 and α-SMA expression. This result also indicates that while a single dose of MP attenuated inflammatory and coagulation disturbances in the acute phase, later phase damage such as fibrosis requires prolonged MP treatment.

We also investigated inflammatory and coagulation changes 28 days after smoke inhalation. We found that proinflammatory changes remained in the lung tissue and blood. However, these parameters were similar in all three MP treatment groups, even though there was a tendency toward a non-statistically significant decrease compared with the S group. These outcomes indicated that the impact of MP on fibrotic changes may not depend on inflammatory changes, but may rely more on profibrotic agents such as TGF-β1 and α-SMA. Because smoke inhalation-induced inflammatory disturbances are complicated, there may be no single ideal agent to solve all the problems that are induced after smoke inhalation, including GCs, which have beneficial effects

and an increased risk of infection with prolonged use.

Animal models of fibrosis have shown that there is an imbalance between thrombosis and fibrinolysis, which is also seen in the alveolar compartment in IPF patients [59]. Extravascular coagulation involving fibrin formation in the intra-alveolar compartment has a pivotal role in the development of pulmonary fibrosis, serving as a provisional matrix for migrating fibroblasts. Additionally, proteases from the coagulation and plasminogen activation systems that respectively form and break down fibrin directly contribute to pulmonary fibrosis [60].

In our study, results of BALF analysis revealed that rats were in the pro-coagulation state for 28 days after smoke inhalation, indicating there was continued fibrin formation. In addition, 3 and 7 days of MP treatment equally and markedly decreased TATc levels in the BALF. Our results demonstrated that MP treatment for 3 and 7 days induced no side effects related to coagulation and fibrinolysis, and decreased pro-coagulant factor production, which may contribute to the reversal of the fibrotic progress.

One main obstacle for prolonged GC management is the increased risk of nosocomial infections secondary to immunosuppression [61]. In our study, inhalation injury impaired endobronchial and alveolar epithelium functions, which resulted in mucosal slough and increased amounts of the debris within the airways, thus reducing the amount and efficacy of ciliary clearance. These problems contribute to the exacerbation of small-airway occlusion, atelectasis, ventilation–perfusion mismatching, and infection after smoke inhalation, which leads to mortality in the later phase of SI-ALI [31]. Therefore, reducing the risk of secondary infection caused by inhalation injury combined with immunosuppression caused by GC treatment is essential for rational drug design and treatment regimens that lead to the effective resolution of inflammation, coagulopathy, and fibrosis in SI-ALI.

Here, we used conscious animals to proximally simulate fire disasters in real life and prevent unwanted side effects, such as disturbed breathing patterns and insufficient model establishment, which may be caused by anesthesia. To exclude potential effects from endogenous GC, we used a smoke group without any treatment and a smoke + Normal Saline group as control groups. All the smoke exposure experiments were carried out at the same time (8:00 am) to match the normal rhythm. We also used three different dosages of methylprednisolone to ensure our results were related to exogenous GC. Indeed, methylprednisolone suppressed basal HPA activity and led to adrenal insufficiency, attenuating the response to stress through negative feedback [62,63], and the rapid suppression of stress-induced corticosterone levels by methylprednisolone was confirmed in blood samples obtained from animals pretreated with different steroid doses [64]. Consequently, we think endogenous GC had little effect in our study.

In the present study, we investigated acute and late inflammatory and fibrotic changes, with the aim of describing respiratory and inflammatory responses following severe smoke exposure. The results are of value for the assessment of acute and long-term health consequences of smoke inhalation injury caused by composite materials. The results from this study also showed the optimal GC dose and duration to treat SI-ALI and later-phase damage, which will minimize the risk of secondary infection caused by GC immunosuppression.

Our study had some limitations. First, our model was characterized by a high mortality rate in the acute phase, especially within 1 h after smoke inhalation. To determine the optimal MP dose, we administered MP intraperitoneally 1 h before smoke inhalation. It is undeniable that the occurrence of a fire disaster and the following inhalation trauma is hard to predict and the objective assessment of the efficiency of GC should carried out after the trauma happens. Because this was a preclinical study, we aimed to use a strategy of MP treatment to determine whether different dosages and durations of treatment would affect the inflammation, coagulation and fibrosis disorders caused by smoke inhalation. Consequently, we chose the most favorable scenario to obtain optimal results, which does not correspond to real-life situations. We would like to highlight that smoke inhalation injury is a special type of trauma, which may increase a victim's caution immediately after fire disaster happens and before smoke has been inhaled. Therefore, it is possible to take preventative action before this kind of trauma occurs, especially for members of special groups including soldiers and firemen, to decrease further injury. This may be a bold vision, but worth further exploration. To simulate real-world conditions, we will focus on verifying the best MP administration regimen after smoke inhalation in future experiments. Second, fibrotic changes caused by smoke inhalation represent a complicated and multifactorial pathological process, and the cellular and molecular mechanisms involved require further investigation, especially the involvement of different origins and phenotypes of macrophages in the modulation of coagulation, inflammation, and fibrosis. We will further investigate how GCs regulate residual, interstitial, and monocyte-derived macrophages in smoke inhalation-induced acute and chronic lung injury.

In conclusion, we identified the capacity of MP to protect against smoke inhalation-induced ALI by attenuating inflammatory infiltrates and subsequent fibrosis after ALI. Exploring the therapeutic patterns showed that a medium dose of MP (4 mg/kg) was involved in the modulation of the acute-phase reaction and a 3-day regimen of MP immediately after smoke inhalation was optimal for inhibiting the progression of fibrosis.

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Declarations of interest

None.

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