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Original Article

Inhibition of matrix metalloproteinase-9 with low-dose doxycycline reduces acute lung injury induced by cardiopulmonary bypass

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Abstract: Objective: This study aims to demonstrate the protective effect of doxycycline, an exogenous inhibitor of matrix metalloproteinases-9 (MMP-9), in the acute lung injury induced by cardiopulmonary bypass (CPB). Methods: A total of 30 healthy mongrel dogs were randomly divided into three groups: Group A (CPB control group, no treatment of doxycycline), Group B (low-dose group, treated with doxycycline at 30 mg/kg) and Group C (high-dose group, doxycycline at 60 mg/kg). The alveolar-arterial oxygen difference (A-aDO_2) and respiratory index (RI) were calculated, the concentration of MMP-9 in plasma was measured by ELISA. The expression levels of MMP-9 was determined by RT-PCR. The lung W/D index was calculated. The myeloperoxidase (MPO) activity of bronchoalveolar lavage fluid (BALF) was measured by colorimetry. The total protein of BALF was measured by Coomassie brilliant blue G-250. The white blood count (WBC) in the sediment of BALF was counted. Results: A-aDO_2, RI, total protein, and MPO activity of BALF, WBC count in BALF sediment and W/D index in group B were significantly lower than that of control group ($P < 0.05$). The concentration of MMP-9 in group C decreased significantly ($P < 0.05$). There were no significant differences in gene expression among the three groups. Conclusion: The results suggested that doxycycline protected the acute lung injury induced by CPB through reducing the concentration of MMP-9 and degradation of the cell membrane, pulmonary neutrophil infiltration and pulmonary edema.

Keywords: Cardiopulmonary bypass, acute lung injury, lung protection, matrix metalloproteinase, doxycycline

Introduction

Almost all patients with cardiopulmonary bypass (CPB) may suffer from lung injury. Some patients show only mild symptoms, while others display severe adult respiratory distress syndrome (ARDS) or acute respiratory failure with high mortality [1]. Acute lung injury (ALI) induced by CPB may be related to the systemic inflammatory response syndrome (SIRS) [2]. Lung, resulting in either ALI or ARDS is one of the most vulnerable target organs of the body which is sensitive to systemic inflammation [3]. The major pathophysiological manifestation of ALI includes impairment of pulmonary capillary basement membrane, damaging of alveolar-capillary barrier, pathological change of vascular endothelial, and increasing of microvascular permeability [4]. Subsequently, inflammatory cells and mediators are created to result in ALI.

The matrix metalloproteinases (MMPs), a large family of Zn^{2+}-dependent and Ca^{2+}-dependent endopeptidases with distinct substrate specificities, degrades most components of the extracellular matrix (ECM) [5]. MMPs play an important role in cardiovascular disease, cancer incidence, and inflammation. Matrix metalloproteinases-9 (MMP-9), also known as gelatinize B, is one of the Zn^{2+}-dependent MMPs involving in the degradation of ECM and inducing the accumulation of neutrophils as a neutrophil chemotactic factor. MMP-9 degrades capillaries and alveolar epithelial basement mem-

brane to result in pulmonary edema and pulmonary shunt, hypoxia by increasing pulmonary capillary permeability and transferring protein-rich plasma into alveoli; thus acute pulmonary injury or ARDS finally are shown [6].

The mechanism of MMP-9 activity in pathogenesis of ALI induced by CBP has been widely discussed [7, 8] and there is also some evidence about the beneficial function of doxycycline, an exogenous inhibitor of MMPs, in other models of ALI [9, 10]. However, it has not been clearly explained whether doxycycline, by inhibiting MMP-9, may play a beneficial role in ALI induced by CBP. This study explored the effect of low-dose doxycycline in the acute lung injury induced by CBP.

Materials and methods

Animals

The experimental plan was audited and approved by Animal Ethics Committee of Anhui Medical University. All experimental procedure and animal care were carried out under the guidance of the Ethics Committee in order to minimize the suffering of animals.

Thirty healthy mongrel puppies, weighing 10-12 kg, were supplied by Animal Laboratory Center of Anhui Medical University. All puppies were placed into a comfortable and quiet room for one week before the initiation of the experimental procedure. Dogs were randomly and averagely divided into three groups. Group A (control group, no treatment of doxycycline), group B (low-dose group, feeding food mixed with doxycycline at 30 mg/kg daily for three days before surgery), and group C (high-dose group, feeding food mixed with doxycycline at 60 mg/kg daily for three days before surgery). Doxycycline (doxycycline hyclate; D-9891; Dox) was purchased from Sigma-Aldrich Corporation.

Surgical preparation

All experimental animals had been fasted for 12 hours and water had been forbidden for 4 hours before the surgical procedure. Anesthesia was maintained with an intravenous injection of pentobarbital sodium (Sigma-Aldrich Corporation, 30 mg/kg) before the surgical procedure. Dogs were then endotracheally intubated and mechanically ventilated. A No. 22 catheter was placed into the right femoral arterial to monitor blood pressure and draw blood sample. The left femoral venous was intubated for fluid infusion and blood drawing.

The dogs were operated with a middle sternotomy incision, and administered intravenous heparin (600 u/kg). When the activated coagulation time (ACT) exceed 480 sec, the pump flow was maintained at 50-80 ml/kg/min, consisting with the intravenous blood volume flowing back to the heart. The aorta was incised at 60 min after the end of CPB and heart rebeating. Subsequently circulation was reestablished in pulmonary artery and 90 min later, the CPB device was removed. Finally, 3 hours after the end of CPB, dogs were sacrificed with 10% KCl.

After anesthesia, blood samples were taken immediately before and after the surgical procedure. Blood samples were taken at the following time points: (a) before operation (0 min), (b) before off-pump (30 min), (c) at the end of CPB (90 min), (d) 1 hour after the end of CPB (150 min), and (e) 3 hour after the end of CPB (270 min).

Bronchoalveolar lavage fluid (BALF)

A complete lobe of the lower right lung was cut, of which bronchial was infused into 20 ml normal saline. Gently washing pump was operated 3 times, and carefully recovered. BALF was then centrifuged at 1000 rpm for 10 min to obtain the supernatant, frozen at -70°C for chemical analysis. The sediment was added with 1 ml normal saline for white blood cell count.

Myeloperoxidase activity

BALF myeloperoxidase (MPO) activity was measured by colorimetry [7]. The kit was purchased from the Institute of Nanjing Bioengineering. MPO activity was defined as alteration in absorbance at 450 nm. The activity of MPO was calculated through following formula: MPO (U/L) = (A254 of sample-A254 of control)/(11.3 × sample volume).

Water content of lung tissue

When sacrificed and a small piece of left lung tissue was taken from each animal and weighed

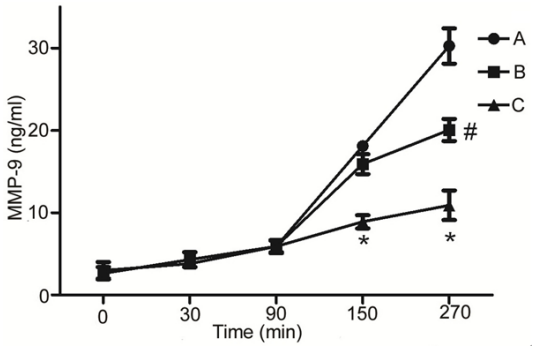

Figure 1. Time-dependent plasma concentration of MMP-9 (vertical-axis, in ng/ml) during CPB surgery. *Compared with Group A, $P < 0.05$; #Compared with Group A and B, $P < 0.05$.

by an analytical balance (wet weight, W). The sample was oven-dried at 65°C for 24 hours and re-weighed. The water content of the lung was defined as a ratio of wet to dry weight (W/D).

Protein content of BALF

The total protein content in BALF was determined by Coomassie brilliant blue G-250 method (Bradford method) [8].

A-aDO_2 and RI

The oxygen left in arterial blood was measured both at the beginning and at the end of the experiment. The alveolar-arterial oxygen difference (A-aDO_2) and respiratory index (RI) were calculated in according to the following formula: A-aDO_2 = PAO2-PaO2, PAO2 = (FIO2 × 713-$PaCO_2$/0.8). RI = A-aDO_2/PaO2. FIO2 stands for the inhaled oxygen concentration (%), PAO2 for alveolar oxygen pressure, PaO2 for arterial partial pressure of oxygen, and $PaCO_2$ for arterial partial pressure of CO_2. The "713" stands for atmospheric pressure 760 minus water vapor pressure 47 (mmHg), and the "0.8" stands for respiratory quotient.

Lung tissue morphology

A small piece of left lung sample was handled with 4% (volume ratio) paraformaldehyde. It was dehydrated, paraffin-embedded, hematoxylin-and eosin-stained (HE), and was subsequently observed by Olympus light microscope.

A small lung sample was taken, cut into 6 pieces (1 mm × 1 mm × 1 mm), and handled quickly with 2.5% solution of glutaraldehyde for more than 8 hours. The sample was immersed in PBS (10 mM, pH 7.4) for 15 min and the process repeated 7 times. After the last immersion, the sample was handled with 1% osmium tetroxide for 1.5 hours, following 30% ethanol for 10 min, 50% ethanol for 15 min, 70% ethanol with uranyl acetate for 8 hours, 80% ethanol for 10 min, 95% ethanol for 15 min, and finally pure ethanol for 45 min. Progressive dehydration of sample was operated during the entire procedure. Finally, instead of ethanol, propylene oxide was used and the sample was embedded with pure epoxy and baked at 40°C for 12 hours and at 60°C for 48 hours, and then sliced by LKB-IV type ultra-thin slicer (Switzerland) into 70 nm thick films. The ultra-thin slices were collected for double staining in the 230-line head of copper acetate uranium and lead citrate for 30 min and 15 min, respectively. The slices were cut off by carbon dioxide. The microcosmic change was observed with Jeol JEM-1230 (Japan) transmission electron microscopy.

ELISA

The plasma concentration of MMP-9 was determined by enzyme-linked immunosorbent assay (ELISA) [9]. The kit was purchased from the United States R&D System Inc., MN, USA. The result of the color depth was positively correlated with MMP-9 concentration of samples. The MMP-9 concentration of each sample was calculated by the absorbance value read from ELISA reader at 450 nm.

Reverse transcription polymerase chain reaction (RT-PCR)

The MMP-9 gene expression of white blood cells was measured by RT-PCR. Samples containing Trizol liquid were separated by trichloromethane, deposited by isopropyl alcohol and scrubbed by ethanol to obtain the total RNA. MMP-9 gene fragments were amplified by RT-PCR. A RNA of 1 μg was used as primer to synthesize the first strand of cDNA by Oligo (dT). A sum of 5 μg product of reverse transcriptase was amplified by PCR. Upstream primer sequence of MMP-9 gene was 5'-GGGCATTCAGGGAGACG-3', and downstream primer sequence was 5'-CGGCCACAAGGAACAGG-3', and β-actin was used as the internal control.

The procedure for the PCR reaction as following: predegeneration for 5 min at 94°C; degen-

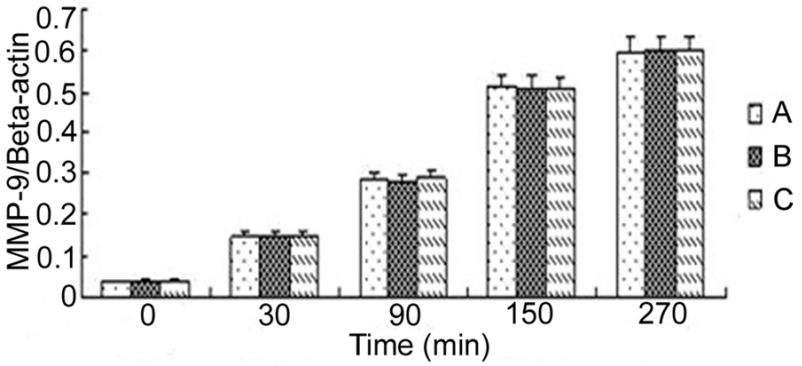

Figure 2. MMP-9 gene expression in plasma during CPB surgery.

Table 1. BALF detection indicators, wet-dry weight index W/D

	Group A (n = 10)	Group B (n = 10)	Group C (n = 10)
MPO (U/L)	51.99 ± 9.62	32.19 ± 7.85#	40.70 ± 11.56#
TP (g/L)	2.04 ± 0.51	1.04 ± 0.31#	1.32 ± 0.78#
WBC (10^9/L)	9.58 ± 1.58	5.51 ± 1.22#	7.13 ± 1.94*
W/D	3.93 ± 0.73	2.87 ± 0.91#	3.65 ± 0.65**

#Compared with Group A, $P < 0.05$; *Compared with Group A and B, $P < 0.05$; **Compared with Group B, $P < 0.05$.

eration for 30 min at 94°C, annealing for 30 min at 51°C, 30 cycles of extension for 30 min at 72°C and amplification extension for 10 min at 72°C. The PCR product was analyzed by 1% agarose gel electrophoresis (5 V/cm × 30 min), photos were taken, and DNA absorbance belt points were measured by a DNA express image analysis software. The MMP-9 relative expression was calculated referring to *β*-actin.

Statistical analysis

Result was analyzed by a statistical software package SPSS12.0 and reported as mean ± S.E.M. Student's *t* test or one-way ANOVA was performed for comparison among groups as indicated. $P < 0.05$ was considered as a significant difference.

Results

MMP-9 plasma concentration

The plasma concentration of MMP-9 increased timely in all three groups at 150 and 270 min after CPB surgery (**Figure 1**). Pretreatment with doxycycline at both 30 mg/kg (low-dose group B) and 60 mg/kg (high-dose group C) decreased the plasma concentration of MMP-9 at 150 and 270 min during CPB surgery. The decreasing of plasma concentration of MMP-9 was more obvious in group C, which was pretreated with higher dose of doxycycline.

Blood MMP-9 gene expression

The MMP-9 mRNA expression in the plasma of untreated control group (group A) during CPB surgery indicated time-dependent increasing of MMP-9 mRNA level. However, pretreatment groups with doxycycline, either at 30 mg/kg or 60 mg/kg did not cause any change in the levels of plasma MMP-9 mRNA (**Figure 2**).

MPO activity in bronchoalveolar lavage fluid

The myeloperoxidase (MPO) activity in BALF of groups pretreated with low-dose of doxycycline decreased significantly (**Table 1**). The MFO activity in BALF of groups pretreated with high-dose of doxycycline also decreased but not significant compared with low-dose group.

Total protein of BALF and white blood cell count in BALF sediment

Total protein (TP) in BALF of group A was significantly higher than that of group B and C. For the sediment of BALF in group A, infiltration of a large number of leukocytes was found, especially for neutrophils, also the WBC count was significantly higher than that in the other two groups (**Table 1**).

Water content of lung tissue

The water content in the lungs was expressed as an index of wet to dry weight ratio (W/D = lung wet weight/lung dry weight). The W/D of group A was the highest, which was significantly higher than that of group B ($P < 0.05$), however no significant difference was found between group C and group A ($P > 0.05$) (**Table 1**).

A-aDO_2 and RI

The A-aDO_2 and RI values in the lungs of all three groups during CPB surgery were all markedly higher than that before the CPB surgery (**Figure 3**). The alveolar-arterial oxygen differ-

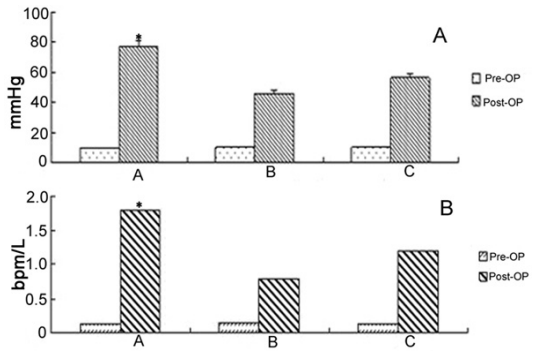

Figure 3. The A-aDO_2 (panel A) and RI (panel B) values in three groups before and after CPB surgery. *P < 0.05.

ence (A-aDO_2) in group A was the highest and was significantly different from those in groups B and C (P < 0.05). The A-aDO_2 value in group C was higher than that in group B, but it was not significant (P > 0.05) (**Figure 3A**). The respiratory index (RI) of group A was the highest and this was significantly different from those in groups B and C (P < 0.05). The respiratory index in group B was lower than that in group C, and the difference was significant (P < 0.05) (**Figure 3B**).

Histological changes

The photomicrographs of the lung tissues handled and derived from the control group (group A) showed diffuse alveolar wall exudative edema and congestive heart telangiectasia (**Figure 4**). Infiltration of a large number of leukocytes and destruction of the alveolar structure can be found in the wall of alveolar and interstitial. The lung tissue derived from the pretreatment group with low-dose of doxycycline (group B), the structure of the alveolar was intact, alveolar space was clear, and there was a relatively little change in inflammation with less swelling and bleeding. The lung tissue derived from the pretreatment groups with high-dose of doxycycline (group C) showed less severe disease than that from group A, but a little more severe than that in group B.

Electron microscopy (**Figure 5**): Thinner type II alveolar epithelial cell (AEC II) in membrane, disappearance of the cell's free surface microvillus, or disappearance of breakdown of mitochondrial swelling crest disorder, and the majority of Lamellar Clear-emptying phenomenon leading to higher vacuole were found from lung tissues in group A. The alveolar epithelial cells from the lung tissues in group B were significantly better than that of group A. The free surface microvillus of cells were arranged regularly, while the mitochondria was mildly swelling and was arranged a little disorderly. In group C, the II-cell lamellar body emptied reduced the formation of a small number of vacuoles, resulting in smaller size. Group C displayed the same disease, less severe than that in group A, but more severe than that in group B.

Discussion

Acute lung injury (ALI) is one of the most common complications of extracorporeal circulation (ECC), usually manifesting non-cardiac and refractory hypoxemia. If the lung function is further worse, it may be deteriorated into acute respiratory distress syndrome (ARDS). Although ARDS incidence of patients after CPB surgery is only 2%, mortality rate of ARDS is 50%. Up to 20% patients require mechanical ventilation for more than 48 hours after CPB surgery [11].

The specific mechanism of ALI during CPB is not clear. It is generally believed that the mechanism may be due to non-physiological contact of blood in CPB, triggering systemic inflammatory response syndrome (SIRS), and leading to accumulation of neutrophils in the lung. Hypoxia, bleeding or ischemia-reperfusion injury may activate neutrophils and release proteolytic enzymes and oxygen free radicals to damage the alveolar-capillary membranes, increase permeability, lung fluid and pulmonary shunt, and finally lead to ALI [12]. Many experimental and clinical observation have proved that accumulation and activation of neutrophil play an important role in the development of acute lung injury during CPB [13, 14]. The protease enzyme released from it is one of the main factors in damaging alveolar capillary membrane, and also the final effective factor of acute lung injury.

The neutrophil elastases, such as plasma protein, matrix metalloproteinase (MMPs) has been considered closely related to acute lung injury and ARDS caused by CPB [15]. The main substrate in degradation of MMP-9 is collagen IV [5], which is the main ingredient of capillary endothelial and alveolar epithelial basement membrane. Therefore, much attention has been paid for the role of MMP-9 in the develop-

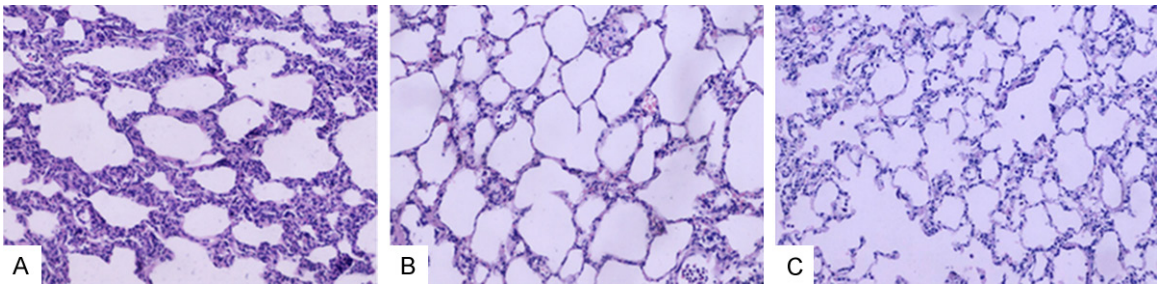

Figure 4. Photomicrographs of lung tissues handled. A, B and C were lung tissues derived from groups A, B, and C, respectively. The lung tissue from group A showed alveolar wall thickening, alveolar and interstitial infiltration of a large number of WBC. The lung tissue from group B showed the structure of the alveolar and leukocyte infiltrating of some WBC. The lung tissue from group C showed an intermediate level of WBC infiltration.

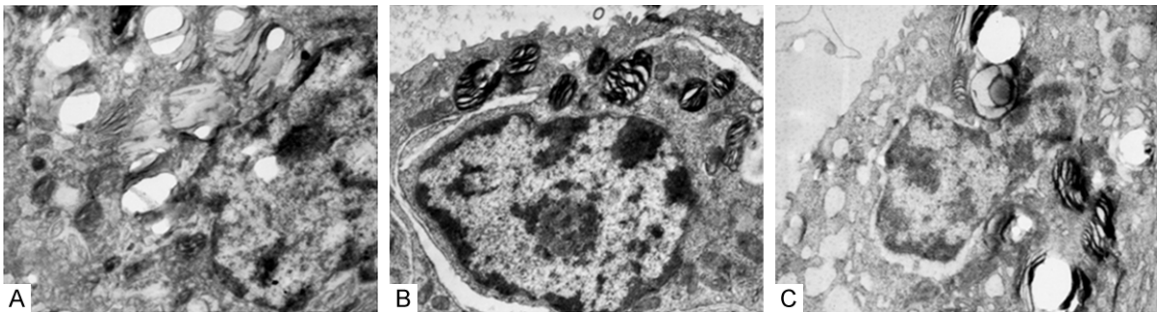

Figure 5. Electron microscopy of fixed lung tissues. A, B and C were lung tissues derived from groups A, B, and C, respectively. The lung tissue of group A showed disintegrated mitochondria of AEC II, apparently emptied lamellar body, and alteration of vacuoles. The lung tissue of group B showed mild swelling of mitochondria and a less emptied lamellar body. The lung tissue of group C showed the disappearance of the partial swelling of the mitochondria ridge and emptying of some lamellar body.

ment of ALI. Recently, many researches have reported that MMP-9 in circulation during CPB increased markedly [16-18] and clearly related to acute lung injury [19-21].

Tissue inhibitor of metalloproteinase (TIMPs), an endogenous inhibitor of MMPs, inhibits all members of the MMPs family. The balance between MMPs and TIMP is one of precondition for maintaining the integrity of extracellular matrix (ECM) [22]. During period of acute lung injury, there is an imbalance between MMPs and TIMPs. TIMPs reduce severity of lung injury [23]. Generally, inhibitors of MMPs can be divided into two categories, endogenous and exogenous. Doxycycline is one of the tetracycline antibiotics. A large number of studies have shown that tetracycline and its modified non-antibiotic analogues are a kind of broad-spectrum exogenous inhibitors of MMPs.

In this study, we used doxycycline to explore the protection of lung injury induced by CBP with dogs are pre-fed with an exogenous MMP inhibitor. Level and activity of MMP-9 were significantly decreased in doxycycline-pretreated group during CPB. The effects of inhibition became more obvious in group pretreated with higher concentration of doxycycline. However, there was no significant difference in mRNA expression among all three groups. The result indicated that doxycycline did not inhibit the gene expression of MMP. Compared with the control group, the severity of lung damage in two doxycycline-pretreated groups was significantly decreased, but the lung injury in high-dose group was more obvious than that in low-dose group. Anyway, the low-dose doxycycline-pretreated group showed better protection against pulmonary injury. Earlier experiments have confirmed that CMT-3 (or COL-3, chemically modified tetracycline) as an inhibitor of MMPs can inhibit the activity of MMPs to decrease concentration of MMP, serum activity and bronchoalveolar lavage fluid. At the same time, by reducing the infiltration of neutrophil to lung tissue and alveolar space, CMT-3 reduced lung injury during CPB surgery [20, 21].

Recently, there are more studies using animals with gene deletion to study matrix metalloproteinase. Warner et al. investigated the role of MMP-9 in acute lung injury through the acute lung injury model of endotoxin, using the MMP-9 mice with gene deletion and wild-type mice (with integrated MMP-9 gene) handled by gene knockout technology. The result has indicated that lung injury of MMP-9-/- mice was significantly decreased compared with MMP-9+/+ mice. Pathological result of lung tissue from MMP-9-/- mice showed alleviation of pulmonary edema. It was believed that MMP-9 mainly increased the permeability of basement membrane of alveolar-capillary to cause pulmonary edema and alveolar bleeding, so the pulmonary shunt increased because of edema and bleeding, and finally acute lung injury was shown [24].

However, on the contrary, two other studies had opposite conclusion. The result of one study showed that MMP-9 plays a protective role in ozone-induced airway inflammation [25]. The study suggested compared with that of MMP9-/- mice during ozone exposure, MMP-9 activity, total protein concentration, neutrophil, and epithelial cells of bronchoalveolar lavage fluid of MMP9+/+ mice significantly increased. The exact mechanism how MMP-9 restricts ozone-induced airway injury is not known, but it may influence the post-transcription of the pro-inflammatory chemokines including KC and MIP-2.

Another one reported that the lack of MMP-9 increased lung injury induced by ventilator [26]. Mice with absence of MMP-9 gene showed more severe lung injury symptom than wild-type mice including decreasing performance of oxygenation, damaging lung dynamics, and leading to more serious pathological damage. The result was consistent with the BALF cell count and the relevant increasing in the activity of MPO, and showed that mechanical ventilation led to the releasing of a large number of neutrophils. By adjusting the regulation of cytokines in response to vacancies, MMP-9 reduced pulmonary neutrophil infiltration, and resisted ventilator-induced lung injury.

In conclusion, this study indicated that doxycycline, an inhibitor of MMPs, protected CPB-induced lung injury. It also showed that a relative low dose of doxycycline was more conducive in protection of the lung compared with that of high-dose. This phenomenon may be explained partly that excessive inhibition of MMP-9 from high dose of doxycycline can cause MMPs/TIMP imbalance, and this is also the shortcoming of our research and in future, we will investigate more details of this topic in further study.

Acknowledgements

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Disclosure of conflict of interest

None.

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