

# PIP, Not FiO<sub>2</sub> Regulates Expression of MMP-9 in the Newborn Rabbit VILI with Different Mechanical Ventilation Strategies

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## ABSTRACT

**Background:** Results from experimental and clinical studies have shown that mechanical ventilation or/and hyperoxia may aggravate a pre-existing lung injury or even cause lung injury in healthy lungs by affecting the expression of MMP-9, but the MMP-9 effects are controversial. How are MMP-9 regulated when multicausative factors of injury such as different FiO<sub>2</sub>, PIP, and respiratory time (RT) impose simultaneously on lungs? **Methods:** Newborn New Zealand white rabbits were randomly allocated to an unventilated air control group or to one of the 2 × 3 × 3 ventilation strategies by using a factorial design, with different FiO<sub>2</sub>, PIP, and RT. Then, lung wet-to-dry ratio (W/D), lung histopathology scores, transmission electron microscope, and cells in BALF were analyzed in these different groups. MMP-9 levels were studied by immunohistochemistry and ELISA. **Results:** MMP-9 levels were significantly different among 3 PIP ventilation regimes (F = 7.215) and MPIP group was the highest among 3 PIP groups. The lung histopathology score in 100% oxygen was significantly higher than in 45% oxygen group (F = 9.037) and MPIP group was the lowest among 3 PIP groups (F = 57.515) and RT 6 h was more serious than RT 1 h. MMP-9 positively correlated with monocytes, but negatively correlated with neutrophils and lung injury histopathology scores. **Conclusions:** Different PIP and FiO<sub>2</sub> exert simultaneously on newborn lung in newborn rabbits ventilation, only mechanical stretch stimulation affects MMP-9 synthesis. Advisable mechanical stretch can promote MMP-9 expression and has protective role in lung in VILI. HPIP causes barotraumas and LPIP induces atelectrauma.

**Keywords:** Mechanical Ventilation; Lung Injury; Matrix Metalloproteinase; Newborn Rabbit; Fraction of Inspired Oxygen; Peak Inspiratory Pressure

## 1. Introduction

Mechanical ventilation (MV) is a life-saving therapy that can also damage the lungs. Matrix Metalloproteinase-9 (MMP-9) can degrade the complex components structure of the lungs and airway, such as extracellular matrix (ECM) and the basement membrane to participate in the lungs and airway reconstruction [1]. On the relationship between MMP-9 and lung injury, there were plenty of studies showing that expression of MMP-9 was regulated by factors of MV [2,3] and high concentrations of oxygen [4] as well as the expression of MMP-9 increases led to lung injury [5,6], but there were also some studies on

protective role in MMP-9 [7,8], absence of MMP-9 worsens mechanical ventilation-induced lung injury (VILI) [9]. These conclusions are based on single-factor condition model and different condition animal models have different experimental results [10]. However, clinically, VILI was multi-factorial, not only including oxygen concentrations, peak inspiratory pressure (PIP), but also including duration of ventilation and so on. To support gas exchange, the parameters about oxygen concentrations and PIP are usually regulated. Importantly, PIP, hyperoxia and duration of ventilation (respiratory time, RT) can induce lung injury, but it has not yet to be determined whether these 3 factors regulated individually

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or simultaneously the expression of MMP-9 causing lung injury when multi-factors impose simultaneously on lungs. Did these factors interact, and/or was one more dominant than the other? Assessment of lung injury was only carried out in animal experiment. We hypothesized that these causative factors of injury could not simultaneously promote MMP-9 producing, otherwise, VILI was impossible to be cured.

## 2. Methods

### 2.1. Ethics

The use of animals was approved by hospital of Beijing Institutional Animal Care and Use Committee (IACUC) and conformed to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

### 2.2. Animals and Experimental Protocol

We employed 114 newborn New Zealand white rabbits (postnatal days, 1 - 5; 44.84 g). The rabbits were randomly allocated to either an unventilated air control group ( $n = 6$ ) or to one of the  $2 \times 3 \times 3$  ventilation strategies by using a factorial design  $FiO_2$ :  $FiO_2 = 100\%$  and  $FiO_2 = 45\%$ ; PIP: high PIP (HPIP) = 25 cmH<sub>2</sub>O, mid PIP (MPIP) = 18 cmH<sub>2</sub>O and low PIP (LPIP) = 10 cmH<sub>2</sub>O; respiratory time (RT): 1 h, 3 h and 6 h; Each group had 6 rabbits, and there were 108 rabbits in the ventilated groups.

### 2.3. Mechanical Ventilation

The rabbits were anesthetized with intraperitoneal sodium pentobarbital, 25 mg/kg. Their body-temperatures were maintained at 39°C by a heating pad. A tracheostomy was performed near the thyroid eminence, and an endotracheal tube ( $1.3 \times 25$  mm<sup>2</sup> intravenous catheter needles) was inserted via tracheostomy (the depth was 1.5 - 2.0 cm), and the endotracheal tube was regulated on the basis of the symmetry of thorax fluctuation after ventilation to avoid atelectasis. Then the rabbits were ventilated (Siemens-900C, Germany) with a fixed positive end-expiratory pressure (PEEP) at 2 cmH<sub>2</sub>O with a respiratory rate of (RR) 50 min<sup>-1</sup>, and an inspiratory time of 0.33 sec at differing levels of  $FiO_2$  and PIP depending on the RT according to a factorial design. No additional fluid support was given in any of the conducted experiments. At the end of the experiment, the rabbits from each experiment group were euthanized at 1, 3, and 6 h with a lethal dose of pentobarbital (100 mg/kg, i.p.). Immediately after sacrifice, lungs were isolated and measurements were performed as described below.

### 2.4. Measurements

The left lung was weighed and subsequently dried for 2

days in an oven at 70°C for estimating the wet-to-dry ratios (W/D). Bronchoalveolar lavage fluid (BALF) was obtained by instilling 1.0 ml saline 3 times by using a T catheter (Abbott, Sligo, Ireland) into the left trachea to lavage the left lung, and approximately 2.7 ml of BALF was retrieved per rabbit. Subsequently, BALF was centrifuged at 2000 g for 10 min, supernatants were snap-frozen in liquid nitrogen for later analysis, and wright-stained smears of cytospin slides of tracheal aspirates were examined for cell density (cells/ × 400 high power field) with white blood cells (WBC) and differential WBC counts (percentage) being done by an observer masked to the group identities.

### 2.5. Lung Histopathology

To analyze the histopathology of the lungs, the right lung lower lobe was fixed in 4% formalin and embedded in paraffin. Sections of 4 μm in thickness were stained with hematoxylin and eosin (HE) and analyzed by a pathologist who was blinded to the group identities. To score lung injury, we used a modified VILI histopathology scoring system as previously described [11,12]. An overall score of VILI was obtained on the basis of the summation of all the scores from air control or ventilated lungs ( $n = 6$  per group).

### 2.6. Electron Microscopy

Electron microscopy was performed to investigate the morphological changes in different ventilation groups. Lung tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 18 h. Lung tissues were post-fixed for 1.5 h in 1% osmium tetroxide (OsO<sub>4</sub>), dissolved in 0.1 M phosphate buffer at pH 7.4, dehydrated in an ascending acetone series, embedded in epon, sectioned at 70 nm, stained with uranyl-acetate and lead nitrate, and examined under an H-7500 transmission electron microscope (HITACHI, Japan).

### 2.7. Matrix Metalloproteinase-9 Assay

At each RT point, the right lung middle lobe was harvested and weighted 0.12 g pulmonary tissue samples and frozen at -70°C until use. The concentrations of MMP-9 in lung tissue homogenate were assayed using a commercially available kit according to the manufacturer's protocol (Rabbit MMP-9 ELISA Kit, Catalog No: E0553Rb, Wuhan EIAab Science.co., Ltd., China; <http://www.eiaab.com>). The concentrations of MMP-9 in lung tissue homogenate were expressed ng·mL<sup>-1</sup>.

### 2.8. Immunohistochemistry

Immunohistochemical analysis of protein expression was performed on paraffin slides with the use of SABC kits

(Boster Biological Technology, Ltd., Wuhan, China), Dako peroxidase kit (Dako, CA) and DAB reagent (DAKO, Denmark) as previously described [13], Tissue sections were incubated with primary antibodies (biotinylated anti rabbit MMP-9) and appropriate secondary antibodies (biotinylated goat anti rabbit). The sections were lightly counterstained with hematoxylin and bound antibody was visualized according to the standard avidin-biotinperoxidase complex protocol with a microscope (Nikon, Japan). The primary antibody was replaced by PBS for negative control slides and the known-positive slice was used as the positive control slides. The immunoreactivities of the lung tissue specimens were scored independently by two pathologists who were blinded to the protocol and experimental groups using the following scheme: the yellow intensity of positive immunoreactivity stained: 0 = no stain; 1 = stramineous; 2 = buffy; 3 = brown and the area of positive yellow stained (0 = 0; 1 = 0 - 1/3; 2 = 1/3 - 2/3; 3 = 2/3 - 1). MMP-9 expression was examined randomly in five HPFs (magnification  $\times 400$ ), and the total scores of the two parts represented the expression of MMP-9.

## 2.9. Statistical Analysis

All data in the results section are expressed as mean  $\pm$  standard deviation.  $2 \times 3 \times 3$  factorial design analysis of variance (ANOVA) was performed. Interaction significant needed to analyze simple effect with one-way ANOVA after fixed certain factor. Post Hoc Test for multiple comparisons, if equal variances assumed, LSD was performed; equal variances not assumed, Tamhane's T2 test was performed. The  $\chi^2$  test was used to compare the distribution of atelectasis. Bivariate correlation was used to determine the correlation of variable. The statistical significance level was set at  $p < 0.05$ .

## 3. Results

### 3.1. FiO<sub>2</sub>, PIP and RT Contribute to W/D. There Are Interactions to W/D between FiO<sub>2</sub> and PIP

Factorial design analysis of variance results show: there were significances in different FiO<sub>2</sub> ( $F = 7.164$ ,  $p = 0.009$ ) and 100% oxygen group was higher than 45% oxygen groups, or in different PIP ( $F = 27.563$ ,  $p = 0.000$ ) groups and 18 cmH<sub>2</sub>O was the lowest among 3 PIP groups, or in different RT groups ( $F = 3.233$ ,  $p = 0.044$ ) and RT6 group was higher than RT1 group ( $p = 0.016$ ). Furthermore, there were interaction effect between FiO<sub>2</sub> and PIP ( $F = 3.674$ ,  $p = 0.029$ ) (R squared = 0.479, Adjusted R squared = 0.381). The simple effect was analyzed. When FiO<sub>2</sub> was 100% or 45%, One way ANOVA showed that 18 cmH<sub>2</sub>O group was the lowest in 3 PIP groups, respectively, ( $p = 0.000$ , 0.010) or ( $p = 0.000$ , 0.000). As

for PIP, there was significance in different FiO<sub>2</sub> groups and 100% oxygen groups was higher than 45% oxygen when PIP was fixed at 25 cmH<sub>2</sub>O ( $F = 4.209$ ,  $p = 0.048$ ) or 18 cmH<sub>2</sub>O ( $F = 10.241$ ,  $p = 0.003$ ), whilst, there was no significance in different FiO<sub>2</sub> groups when PIP was fixed at 10 cmH<sub>2</sub>O ( $F = 0.270$ ,  $p = 0.607$ ) (Table 1).

### 3.2. PIP, RT 2 Factors Contribute to WBCs in BALF. There Are Interactions to Cells between FiO<sub>2</sub> and RT as well as PIP and RT

Factorial design ANOVA (Table 2) showed that the number of WBCs in BALF was similar in the 2 FiO<sub>2</sub> groups ( $F = 0.122$ ,  $p = 0.728$ ), whereas PIP ( $F = 78.437$ ,  $p < 0.001$ ) and RT ( $F = 9.114$ ,  $p < 0.001$ ) had a significant effect on the number of WBCs. Moreover, there were interaction effects between FiO<sub>2</sub> and RT ( $F = 6.206$ ,  $p = 0.003$ ) or between PIP and RT ( $F = 3.468$ ,  $p = 0.011$ ) (R Squared = 0.693, Adjusted R Squared = 0.636). The simple effect was analyzed. when FiO<sub>2</sub> was 100%, ONE-WAY ANOVA showed that there were no significance in cells among RT 1 h, 3 h and 6 h groups ( $F = 2.386$ ,  $p = 0.102$ ), but fixed FiO<sub>2</sub> was 45%, there were significance in cells among RT 1 h, 3 h and 6 h groups ( $F = 3.481$ ,  $p = 0.038$ ), multiple comparisons with Tamhane were no significance. when fixed RT was 3 h, 100% oxygen group was lower than 45% oxygen group in cells ( $F = 5.393$ ,  $p = 0.026$ ). But there were no significance when fixed RT was 1 h ( $F = 0.051$ ,  $p = 0.822$ ) and 6 h ( $F = 1.027$ ,  $p = 0.318$ ). When fixed PIP was 25 cmH<sub>2</sub>O, ONE-WAY ANOVA showed that there were significance in CELLS among RT 1 h, 3 h, 6 h groups ( $F = 3.923$ ,  $p = 0.030$ ) and RT 6 h group was the highest compared with RT 1 h and 3 h ( $p = 0.024$ , 0.019). When fixed PIP was 18 cmH<sub>2</sub>O, ONE-WAY ANOVA showed that there were significance in cells among 3 RT groups ( $F = 8.862$ ,  $p = 0.001$ ) and RT 3 h group was the highest compared with RT 1 h and 6 h ( $p = 0.000$ , 0.013). When fixed PIP was 10 cmH<sub>2</sub>O, ONE-WAY ANOVA showed that there were significance in cells among 3 RT groups ( $F = 6.611$ ,  $p = 0.004$ ) and RT 1 h group was the lowest compared with RT3 h and 6 h ( $p = 0.001$ , 0.040). As for fixed RT 1 h, 3 h and 6 h, ONE-WAY ANOVA showed that there were significance in cells among 3 PIP groups, ( $F = 39.001$ , 11.188, 33.732, respectively,  $p = 0.000$ ) and 25 cmH<sub>2</sub>O group was the highest compared with 18 and 10 cmH<sub>2</sub>O group in fixed RT 1 h, 3 h and 6 h.

### 3.3. FiO<sub>2</sub>, PIP, RT 3 Factors Contribute to Neutrophil in BALF. There Are Interactions to Neutrophil between FiO<sub>2</sub> and PIP as well as PIP and RT

Factorial design ANOVA showed that the neutrophil levels were significantly different in these ventilation regimes (Table 2). These results show that neutrophil le-

**Table 1. W/D in different ventilation groups.**

RT	100% oxygen				45% oxygen			
	HPIP	MPIP	LPIP	Total	HPIP	MPIP	LPIP	Total
1 h	5.65 ± 0.16	5.54 ± 0.22	5.59 ± 0.24	5.59 ± 0.20	5.52 ± 0.16	5.16 ± 0.14	5.62 ± 0.29	5.43 ± 0.28
3 h	5.79 ± 0.26	5.47 ± 0.15	5.73 ± 0.23	5.66 ± 0.25	5.59 ± 0.27	5.32 ± 0.21	5.74 ± 0.22	5.55 ± 0.29
6	5.87 ± 0.09	5.43 ± 0.37	5.73 ± 0.19	5.68 ± 0.30	5.77 ± 0.19	5.25 ± 0.22	5.81 ± 0.22	5.61 ± 0.33
Total	5.77 ± 0.20	5.48 ± 0.25	5.68 ± 0.22	5.64 ± 0.25	5.62 ± 0.23	5.24 ± 0.19	5.72 ± 0.25	5.53 ± 0.30

**Table 2. Cells count and cells classification in BALF in different ventilation groups.**

RT	100% oxygen				45% oxygen			
	HPIP	MPIP	LPIP	Total	HPIP	MPIP	LPIP	Total
<b>Cells in BALF</b>								
1 h	26.08 ± 4.82	12.26 ± 3.00	9.08 ± 1.53	15.80 ± 8.23	26.59 ± 7.89	10.71 ± 2.33	12.12 ± 7.01	16.47 ± 9.43
3 h	29.63 ± 10.10	19.98 ± 3.09	16.88 ± 2.49	22.16 ± 8.11	22.36 ± 6.79	14.73 ± 3.63	12.62 ± 2.98	16.57 ± 6.22
6 h	31.57 ± 7.18	11.85 ± 3.76	16.65 ± 5.61	20.02 ± 10.16	37.34 ± 11.22	15.48 ± 1.23	18.55 ± 5.63	23.79 ± 12.07
Total	29.09 ± 7.58	14.69 ± 4.94	14.20 ± 5.07	19.33 ± 9.11	28.77 ± 10.54	13.64 ± 3.25	14.43 ± 5.95	18.94 ± 9.98
<b>Neutrophils in BALF</b>								
1 h	32.83 ± 10.11	8.00 ± 2.61	19.17 ± 5.34	20.00 ± 12.23	26.50 ± 5.01	8.17 ± 4.12	17.00 ± 5.14	17.22 ± 8.91
3 h	21.67 ± 13.69	10.33 ± 4.03	12.17 ± 2.32	14.72 ± 9.36	12.33 ± 2.58	9.17 ± 2.32	12.67 ± 4.23	11.39 ± 3.38
6 h	16.67 ± 3.39	8.17 ± 2.64	26.00 ± 6.03	16.94 ± 8.50	5.33 ± 1.86	9.17 ± 2.93	18.83 ± 4.79	11.11 ± 6.67
Total	23.72 ± 11.70	8.83 ± 3.17	19.11 ± 7.38	17.22 ± 10.20	14.72 ± 9.61	8.83 ± 3.05	16.17 ± 5.18	13.24 ± 7.18
<b>Monocytes in BALF</b>								
1 h	37.83 ± 9.15	64.17 ± 7.39	17.00 ± 4.15	39.67 ± 20.98	23.17 ± 2.99	62.00 ± 11.19	50.33 ± 5.89	45.17 ± 18.16
3	34.50 ± 13.81	49.50 ± 12.23	31.67 ± 10.56	38.56 ± 14.06	31.17 ± 5.71	64.67 ± 4.84	29.83 ± 6.37	41.89 ± 17.42
6 h	19.00 ± 9.30	47.00 ± 18.06	41.33 ± 6.56	35.78 ± 16.99	23.83 ± 11.09	65.00 ± 7.56	41.00 ± 6.32	43.28 ± 19.15
Total	30.44 ± 13.32	53.56 ± 14.72	30.00 ± 12.51	38.00 ± 17.32	26.06 ± 7.89	63.89 ± 7.90	40.39 ± 10.40	43.44 ± 17.96
<b>Lymphocytes in BALF</b>								
1 h	29.33 ± 10.29	27.83 ± 7.22	63.83 ± 7.11	40.33 ± 18.82	50.33 ± 4.03	29.83 ± 8.98	32.67 ± 6.83	37.61 ± 11.37
3 h	43.83 ± 12.02	40.00 ± 10.45	56.17 ± 12.02	46.67 ± 12.94	56.50 ± 5.17	26.17 ± 4.54	57.50 ± 9.59	46.72 ± 16.27
6 h	67.33 ± 5.85	44.83 ± 16.53	32.67 ± 5.39	48.28 ± 17.82	70.83 ± 10.09	25.83 ± 5.34	40.17 ± 8.04	45.61 ± 20.75
Total	46.83 ± 18.53	37.56 ± 13.49	50.89 ± 15.88	45.09 ± 16.77	59.22 ± 10.98	27.28 ± 6.45	43.44 ± 13.20	43.31 ± 16.77

vels were correlated with  $FiO_2$ , PIP, and RT ( $F = 14.405$ ,  $37.958$ ,  $10.665$ , respectively,  $p < 0.001$ ). 100% oxygen group was higher than 45% oxygen group in neutrophil ( $F = 14.405$ ,  $p = 0.000$ ). 18  $cmH_2O$  group was the lowest compared with 25 and 10  $cmH_2O$  group in neutrophil ( $p = 0.000$ ,  $0.000$ ) and RT 1 h group was higher than RT 3 h group in neutrophil ( $p = 0.035$ ). Furthermore, there were interaction effects between  $FiO_2$  and PIP ( $F = 6.378$ ,  $p = 0.003$ ) or between PIP and RT ( $F = 18.228$ ,  $p < 0.001$ ) (R Squared = 0.692, Adjusted R Squared = 0.633). The simple effect was analyzed. When fixed  $FiO_2$  was 100%, 18  $cmH_2O$  group was the lowest compared with 25 and 10  $cmH_2O$  group in neutrophil ( $p = 0.000$ ,  $0.000$ ). When fixed  $FiO_2$  was 45%, 18  $cmH_2O$  group was the lower than 10  $cmH_2O$  group in neutrophil ( $p = 0.000$ ). when fixed PIP was 25  $cmH_2O$ , 100% oxygen group was the higher than 45% oxygen group in neutrophil ( $p = 0.017$ ), but there were no significance between 100% oxygen group and 45% oxygen group when fixed PIP was 10  $cmH_2O$  or 18  $cmH_2O$ . When fixed PIP was 25  $cmH_2O$ ,

RT 1 h group was the highest compared with RT 6 h and 3 h group in neutrophil ( $p = 0.001$ ,  $0.000$ ). When fixed PIP was 10  $cmH_2O$ , RT 6 h group was the highest and RT 3 h was the lowest compared with RT 1 h, 3 h and 6 h group in neutrophil ( $p = 0.010$ ,  $0.000$ ,  $0.045$ ), but there were no significance among 3 RT groups when fixed PIP was 18  $cmH_2O$ . When fixed RT was 1 h, the 25  $cmH_2O$  group was the highest and 18  $cmH_2O$  group was the lowest among 3 PIP groups in neutrophil ( $p = 0.000$ ,  $0.000$ ,  $0.000$ ). When fixed RT was 6 h, the 10  $cmH_2O$  group was the highest compared with 25  $cmH_2O$  and 18  $cmH_2O$  group in neutrophil ( $p = 0.001$ ,  $0.000$ ), but there were no significance among 3 PIP groups in neutrophil. When fixed RT was 3 h.

### 3.4. $FiO_2$ , PIP 2 Factor Contribute to Monocytes in BALF, There Are Interactions to Monocytes between $FiO_2$ and PIP, PIP and RT as well as $FiO_2$ , PIP and RT

Factorial design ANOVA showed that the monocytes

counts in 100% oxygen group were lower than that of 45% oxygen group ( $F = 9.305$ ,  $p = 0.003$ ). There were significance in 3 PIP groups ( $F = 106.749$ ,  $p = 0.000$ ) and 18 cmH<sub>2</sub>O group was the highest than 25 and 10 cmH<sub>2</sub>O ( $p = 0.000$ ,  $0.000$ ), but RT were no difference ( $F = 0.952$ ,  $p = 0.390$ ). Furthermore, there were interaction effects between FiO<sub>2</sub> and PIP ( $F = 7.588$ ,  $p = 0.001$ ) or between PIP and RT ( $F = 5.092$ ,  $p = 0.001$ ) or among PIP, RT and FiO<sub>2</sub> (R Squared = 0.771, Adjusted R Squared = 0.728). The simple effect was analyzed. When fixed FiO<sub>2</sub> was 100%, 18 cmH<sub>2</sub>O group was the highest compared with 25 and 10 cmH<sub>2</sub>O groups in monocytes ( $p = 0.000$ ,  $0.000$ ). When fixed FiO<sub>2</sub> was 45%, 18 cmH<sub>2</sub>O group was the highest and 25 cmH<sub>2</sub>O group was the lowest among 3 PIP groups in monocytes ( $p = 0.000$ ,  $0.000$ ,  $0.000$ ). When fixed PIP was 18 cmH<sub>2</sub>O or 10 cmH<sub>2</sub>O, 45% oxygen group was higher 100% oxygen group in monocytes ( $F = 6.887$  or  $7.339$ ,  $p = 0.013$  or  $0.010$ ), but 25 cmH<sub>2</sub>O group was no difference. As for interaction effects between PIP and RT, when fixed PIP was 25 cmH<sub>2</sub>O, RT 3 h was higher than RT 6 h in monocytes ( $p = 0.009$ ); When fixed PIP was 10 cmH<sub>2</sub>O, RT 6 h was higher than RT 3 h in monocytes ( $p = 0.007$ ); however, there was no difference among 3 RT group in monocytes. When fixed RT 1 h or 3 h, 18 cmH<sub>2</sub>O group was the highest among 3 PIP groups (all of  $p$  were  $0.000$ ); When fixed RT 6 h, 18 cmH<sub>2</sub>O group was the highest and 25 cmH<sub>2</sub>O was the lowest among 3 PIP groups ( $p = 0.000$ ,  $0.000$ ,  $0.030$ ) (**Table 2**).

### 3.5. PIP and RT Contributes to Lymphocytes. There Are Interactions to Lymphocytes between FiO<sub>2</sub> and PIP, PIP and RT as well as FiO<sub>2</sub>, PIP and RT

Factorial design ANOVA showed that the lymphocytes counts in 100% and 45% oxygen group were no difference ( $F = 1.081$ ,  $p = 0.301$ ). There were significance in 3 PIP groups ( $F = 51.462$ ,  $p = 0.000$ ) and 18 cmH<sub>2</sub>O group was the lowest among 3 PIP groups ( $p = 0.000$ ,  $0.000$ ). RT were difference ( $F = 9.366$ ,  $p = 0.000$ ), but Post Hoc Test for 3 RT groups were no difference with Tamhane ( $p = 0.092$ ,  $0.157$ ,  $1.000$ ). Furthermore, there were interaction effects between FiO<sub>2</sub> and PIP ( $F = 17.386$ ,  $p = 0.001$ ) or between PIP and RT ( $F = 20.852$ ,  $p = 0.000$ ) or among FiO<sub>2</sub>, RT and PIP ( $F = 11.785$ ,  $p = 0.000$ ). (R Squared = 0.762, Adjusted R Squared = 0.717) (**Table 2**).

### 3.6. FiO<sub>2</sub>, RT, PIP 3 Factors Contribute to Lung Injury Histopathology Scores

Factorial design ANOVA showed that the lung histopathology scores in 100% oxygen was significantly higher than in 45% oxygen group ( $F = 9.037$ ,  $p = 0.003$ ) (**Table**

**3**) and 18 cmH<sub>2</sub>O group lung histopathology scores was the lowest among 3 PIP groups ( $F = 57.515$ ,  $p < 0.000$ ). RT were significant differences ( $F = 3.586$ ,  $p = 0.032$ ) and RT 6 h groups was higher than RT 1 h group ( $p = 0.010$ ). However, there were no interaction effects among FiO<sub>2</sub>, RT and PIP.

### 3.7. PIP Contributes to MMP-9 Levels. There Was Interaction to MMP-9 between FiO<sub>2</sub> and PIP in Lung Tissue Bomogenate

Factorial design ANOVA showed that the MMP-9 levels were significantly different among 3 PIP ventilation regimes ( $F = 7.215$ ,  $p = 0.932$ ) and 18 cmH<sub>2</sub>O groups was the highest than the other 2 PIP groups ( $p = 0.000$ ,  $0.008$ ), but there were no significance between the 25 and 10 cmH<sub>2</sub>O. FiO<sub>2</sub> and RT did not contribute to MMP-9 ( $F = 0.007$ ,  $0.401$ ;  $p = 0.932$ ,  $0.671$ , respectively). There were interaction to MMP-9 between FiO<sub>2</sub> and PIP. When fixed PIP was 25 cmH<sub>2</sub>O, there were no significance between 100% and 45% oxygen group in MMP-9 ( $F = 0.583$ ,  $p = 0.450$ ). When fixed PIP was 18 cmH<sub>2</sub>O, 100% oxygen group was higher than 45% oxygen group in MMP-9 ( $F = 4.403$ ,  $p = 0.043$ ). When fixed PIP was 10 cmH<sub>2</sub>O, 100% oxygen group was lower than 45% oxygen group in MMP-9 ( $F = 4.392$ ,  $p = 0.044$ ). when fixed FiO<sub>2</sub> was 100%, there were significances among 3 PIP groups ( $F = 10.622$ ,  $p = 0.000$ ) and the 18 cmH<sub>2</sub>O group was the highest than the others PIP groups ( $p = 0.002$ ,  $0.003$ ) in MMP-9, ut there was no significance between 25 and 10 cmH<sub>2</sub>O. There were no significance among 3 PIP groups in MMP-9 ( $F = 1.248$ ,  $p = 0.296$ ) when fixed FiO<sub>2</sub> was 45% (**Table 4**).

### 3.8. Pathology

Ten of 108 rabbits were induced with pulmonary atelectasis (**Figure 1(c)**, **Table 5**), and PIP ( $\chi^2 = 6.834$ ,  $p < 0.05$ ) or RT ( $\chi^2 = 8.154$ ,  $p < 0.05$ ) induced atelectasis significantly, but FiO<sub>2</sub> did not ( $\chi^2 = 0.441$ ,  $p > 0.05$ ). Although the histopathological changes in the ventilation groups were greatly different from the control groups (**Figure 2(a)**), they shared the common structural changes among these ventilation group. Change in lung structure with patchy areas of parenchymal thickening and small airspaces interspersed with areas of enlarged airspaces, with inflammatory cell infiltrated. Pathological features from the exudative phase to the early proliferative phase of diffuse alveolar damage such as: epithelial destruction, capillary congestion, interstitial oedema, intra-alveolar oedema, haemorrhage, mononuclear infiltration, polymorphonuclear infiltration, interlobular septal thickening, hyaline membrane formation, uneven alveolar ventilation and microatelectasis were observed in the present experimental groups. The hemorrhage in the 100% oxygen

**Table 3. Lung injury histopathology scores in different ventilation groups.**

RT	100% oxygen				45% oxygen			
	HPIP	MPIP	LPIP	Total	HPIP	MPIP	LPIP	Total
1 h	7.67 ± 1.21	4.33 ± 1.03	7.50 ± 1.05	6.50 ± 1.89	7.17 ± 0.98	4.33 ± 1.03	5.67 ± 1.03	5.72 ± 1.53
3 h	8.17 ± 1.47	4.50 ± 0.84	8.00 ± 2.00	6.89 ± 2.25	7.67 ± 1.75	4.17 ± 0.41	5.83 ± 1.60	5.89 ± 1.97
6 h	8.33 ± 2.07	5.67 ± 0.8	7.67 ± 1.21	7.22 ± 1.80	8.17 ± 1.94	4.50 ± 0.55	7.33 ± 1.63	6.67 ± 2.14
Total	8.06 ± 1.55	4.83 ± 1.04	7.72 ± 1.41	6.87 ± 1.97	7.67 ± 1.57	4.33 ± 0.69	6.28 ± 1.56	6.09 ± 1.91

**Table 4. MMP-9 assay in lung tissue homogenate.**

RT	100% oxygen				45% oxygen			
	HPIP	MPIP	LPIP	Total	HPIP	MPIP	LPIP	Total
1 h	61.09 ± 13.68	93.46 ± 27.08	59.38 ± 10.78	71.31 ± 23.77	69.77 ± 10.02	76.72 ± 21.78	89.38 ± 26.94	78.62 ± 21.27
3 h	77.45 ± 13.14	89.78 ± 25.80	67.36 ± 12.46	78.20 ± 19.53	72.74 ± 12.36	86.93 ± 18.49	70.69 ± 12.67	76.79 ± 15.74
6 h	66.41 ± 16.99	102.39 ± 25.09	75.81 ± 30.28	81.54 ± 28.03	73.31 ± 17.31	72.89 ± 25.55	83.64 ± 12.90	76.61 ± 18.85
Total	68.32 ± 15.49	95.21 ± 25.03	67.52 ± 19.93	77.02 ± 23.96	71.94 ± 12.85	78.84 ± 21.66	81.24 ± 19.35	77.34 ± 18.42

**Table 5. Pulmonary atelectasis in different ventilation groups (n/group).**

	PIP			RT			FiO <sub>2</sub>	
	HPIP	MPIP	LPIP	1 h	3 h	6 h	100%	45%
Atelectasis	1	2	7	0	3	7	4	6
Normal	35	34	29	36	33	29	50	48
$\chi^2$		6.834			8.154		0.441	
p		<0.05			<0.05		>0.05	

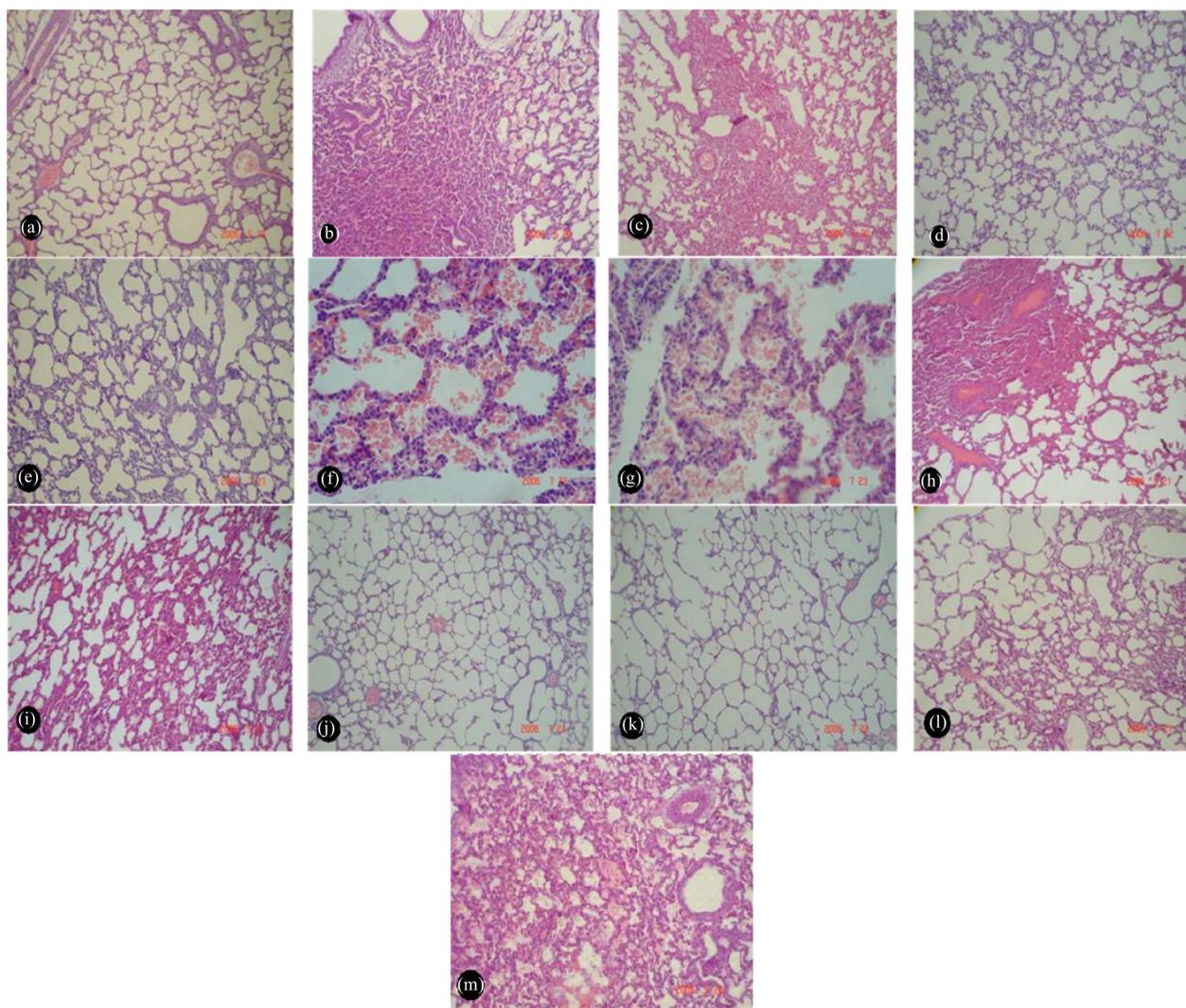
**Figure 1. Gross appearance of lung tissue.**

ventilation groups was more serious than that in the 45% oxygen ventilation groups (**Figures 1(a)** and **(b)**). Uneven alveolar sizes, microatelectasis, significant hyaline membrane formation, interlobular septal thickening and interlobular septal destruction were obviously observed in LPIP ventilation groups (**Figures 1** and **2(b),(c), (h),(i)**). Pulmonary hemorrhage, significant pulmonary bullae formation and the hemorrhage were obviously observed not only within the interlobular septal and alveolar spaces but also within bronch-walls in HPIP ventilation groups, but the atelectasis were less observed (**Figures 2 (f),(g),(l),(m)**). Compared with the HPIP ventilation groups and LPIP ventilation groups, the pathological changes in the MPIP groups were better (**Figure 1(b)**; **Figures 2(d),(e),(k),(l)**): alveolar distention even, pulmonary hemorrhage, intra-alveolar oedema, atelecta-

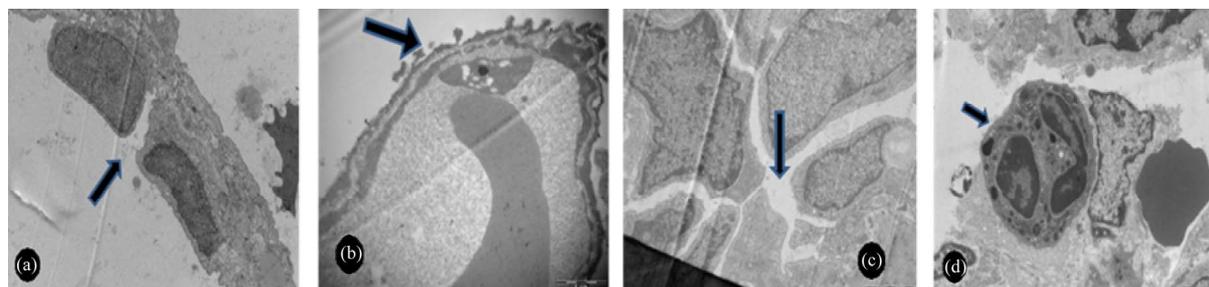
sis, the hyaline membrane formation and pulmonary bullae were decreased significantly. To further confirm our results, we performed transmission electron microscope to illustrate the lung structural features. Lung tissue without ventilation had the continuous vascular endothelial cells and the integrity basement membrane. But with the ventilation going, disappeared and collapse cell conjunction were found in HPIP groups (**Figure 3(a)**) and microatelectasis were found in LPIP groups (**Figure 3(c)**). The lung structural or air-blood barrier of MPIP groups were normal (**Figure 3(b)**).

### 3.9. Immunohistochemical Detection the Express of MMP-9 in Lung Tissue (**Figure 4**)

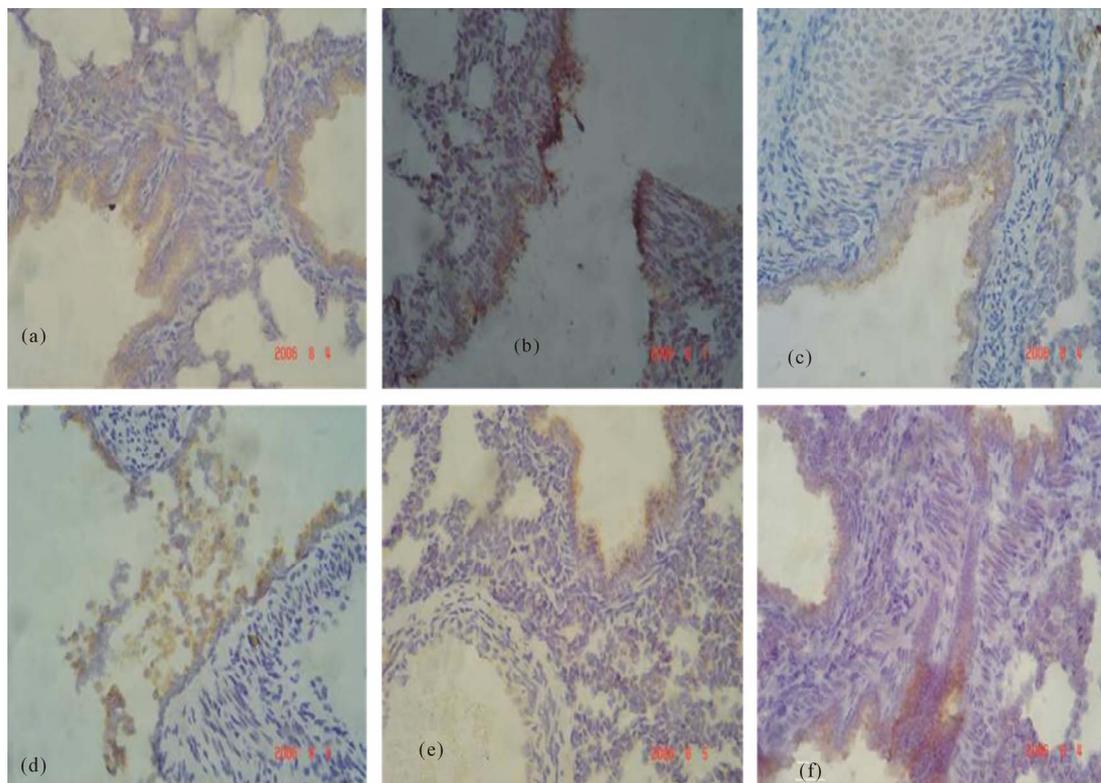
Positivism MMP-9-expression was observed in alveolar



**Figure 2.** Microscopic changes in Hematoxylin-eosin (H&E) staining lungs tissues. No ventilation control group (a); 100% oxygen LPIP ventilation for 1 h (b) and 100% oxygen LPIP ventilation for 6 h (c); 45% oxygen LPIP ventilation for 1 h (h), 45% oxygen LPIP ventilation for 6 h (i) show the evidence of extensive lung injury with microatelectasis, hyaline membrane formation and interlobular septal thickening. 100% MPIP ventilation for 1 h (d) and 100% oxygen MPIP ventilation for 6 h (e); 45% oxygen MPIP ventilation for 1 h (j) and 45% oxygen MPIP ventilation for 6 h (k) illustrate the pathological changes in the moderate pressure groups are better: alveolar distention are even; After 1 h, 6 h 100% oxygen HPIP (f), (g) and 45% oxygen HPIP (l), (m) ventilate, severe infiltration of inflammatory cells into the interstitium, hyaline membrane formation, severe haemorrhage, and pulmonary bullae are observed.



**Figure 3.** Transmission electron microscope changes in lungs tissues. (a) 45% oxygen and HPIP ventilation for 1 h, magnification  $\times 10000$ ; (b) 100% oxygen MPIP 6 h magnification  $\times 3000$ ; (c) 45% oxygen and LPIP ventilation for 6 h, magnification  $\times 15000$ , atelectasis alveolar space. (d) 100% oxygen LPIP 6 h, alveolar neutrophilic infiltration (magnification  $\times 3000$ ).



**Figure 4.** Immunohistochemical localization of MMP-9 in lung tissue sections. (a): Normal control lung tissue (magnification,  $\times 200$ ). (b): Strong expression of MMP-9 in Ventilation 1 h with 100% oxygen and MPIP. (c): Weak expression of MMP-9 in Ventilation 3 h with 100% oxygen and HPIP. (d): MMP-9 expressed increase in ventilation 6 h with 45% oxygen and LPIP, (e): Less normal control lung tissue expression of MMP-9 in ventilation 6 h with 100% oxygen and HPIP. (f): Almost normal control lung tissue of MMP-9-expression in ventilation 1 h with 45% oxygen and HPIP.

macrophages, alveolar lining epithelium, alveolar septal interstitium, and interstitium cells in unventilated rabbits (**Figure 4(a)**). Strong expression of MMP-9 was detected in ventilation for 1 h with 100% oxygen and MPIP (**Figure 4(b)**). In newborn rabbits Ventilation for 3 h with 100% oxygen and HPIP, Weak expression of MMP-9 was detected in alveolar lining epithelium and inflammatory cells (**Figure 4(c)**). After 6 h ventilation with 45% oxygen and LPIP, MMP-9 was strong expressed in alveolar macrophages, neutrophils, and alveolar lining epithelium. Injury and defluxion airway epithelium mucosae was also observed (**Figure 4(d)**). In ventilation for 6 h with 100% oxygen and HPIP, less normal control lung tissue expression of MMP-9 was detected (**Figure 4(e)**). However, in ventilation for 1 h with 45% oxygen and HPIP, it was almost normal control lung tissue of MMP-9-expression in alveolar lining epithelium and inflammatory cells (**Figure 4(f)**).

### 3.10. MMP-9 Positively Correlated with Monocytes, but Negatively Correlated with Neutrophils, Lung Injury Histopathology Scores

To understand the relationship between these variables in

the different ventilation regimes, pearson correlation analysis was performed. The results revealed that MMP-9 positively correlated with monocytes in BALF ( $r = 0.262$ ,  $p = 0.006$ ), MMP-9 negatively correlated with neutrophils in BALF ( $r = -0.235$ ,  $p = 0.014$ ), lung injury histopathology scores ( $r = -0.280$ ,  $p = 0.003$ ). However, there were no relationship between MMP-9 and W/D ( $r = -0.021$ ,  $p = 0.827$ ), Cells ( $r = -0.067$ ,  $p = 0.494$ ), lymphocytes ( $r = -0.150$ ,  $p = 0.122$ ) in BALF.

W/D positively correlated with lung injury histopathology scores ( $r = 0.462$ ,  $p = 0.000$ ). Cells ( $r = 0.322$ ,  $p = 0.001$ ), lymphocytes ( $r = 0.409$ ,  $p = 0.000$ ) in BALF, but negatively correlated with monocytes in BALF ( $r = -0.460$ ,  $p = 0.000$ ).  $n = 108$ .

## 4. Discussion

Our study indicated that atelectasis increases significantly in 10 cmH<sub>2</sub>O PIP ventilation groups and RT 6 h groups, but different oxygen has no effect on atelectasis. These also confirmed that the lower PIP, the easier to induce uneven alveolar ventilation. W/D and the lung histopathology scores were positive relationship ( $r = 0.462$ ,  $p = 0.000$ ) and they were the marker of lung injury. Different FiO<sub>2</sub>, PIP and RT could cause lung injury and

the degree of lung injury in 100% oxygen groups was more severe than in 45% oxygen groups. Lung injury in RT 6 h groups was more severe than in RT 1 h groups. The fact that lung injury in MPIP group was the lightest among 3 PIP groups was confirmed by W/D, the lung histopathology scores and lung histopathology. There was significant pulmonary hemorrhage in pulmonary alveoli and bronch-walls in HPIP groups, so only MPIP caused uniform alveolar distention (**Figure 1(b)**), HPIP was easy to cause barotraumas (**Figure 1(a)**) [14,15] and LPIP was easy to atelectasis (**Figure 1(c)**).

MMP-9 is a metalloproteinase secreted by a wide variety of cell types. In the lung, MMP-9 is synthesized by normal resident structural and inflammatory cells such as bronchial epithelial cells [16], alveolar epithelial cells [17], and alveolar macrophages [18]. All of these cell types can greatly increase their MMP-9 secretion after stimulation [16-18]. In our experiment, the cell count and cell classification were researched in BALF and the results found that MMP-9 was not correlated with the cell count, lymphocytes and W/D. It is agreed with Gushima report that MMP-9 expression was not correlated with the number of total cells or lymphocytes [19], MMP-9 was negatively correlated with lung histopathology scores and neutrophils, but MMP-9 was positively correlated with alveolar macrophages. On the basis of these findings, it has been suggested that MMP-9 may derive from alveolar macrophages. Macrophages are a type of inflammatory cell that synthesizes hundreds of bioactive substances and enzymes. Macrophages are sensitive to cyclic pressure stretching and pressure-stretching stimulus. Macrophages respond to pressure-stretching strain by secreting MMP-9 and the chemokine IL-8 [20]. All of these results (factorial design ANOVA, lung histopathology and immunohistochemistry) confirmed that the higher alveolar macrophages, the higher the level of MMP-9, the lower alveolar neutrophilic granulocyte, the lighter lung injury. MMP-9 is the production of macrophages. Normally, protected lung cell is alveolar macrophages, but not neutrophilic granulocyte [21]. Absence of MMP-9 led to a more severe injury with neutrophil increase in the alveolar spaces in 100% oxygen LPIP 6 h (**Figure 3(d)**). It appeared that MMP-9 has advantage over VILI. Mice lacking MMP-9 developed more severe lung damage after high-pressure ventilation than their wildtype counterparts [9], and MMP-9 deficiency worsened lung injury in a model of bronchopulmonary dysplasia [8]. MMP-9 had protective role in O<sub>3</sub>-induced lung neutrophilic inflammation and hyperpermeability. MMP-9 deficiency was associated with enhanced airway epithelial injury and neutrophil recruitment [7]. MMP-9 deficiency impairs host defense against abdominal sepsis [22]. Other authors have shown a similar protective role in MMP-9 in different models of lung injury [7-9,22-24].

It is known that proteolytic function of MMP-9 affects cytokine and chemokine levels as well as their activities. MMP-9 could cleave different cytokine and chemokines, like IL-1 $\beta$  [25]. MMP-9 protected against ventilator-induced lung injury by decreasing alveolar neutrophilic infiltration, probably by modulation of the cytokine response in the air spaces [9]. MMP-9 was first identified in neutrophils and could also be expressed by neutrophils [26], but neutrophils-derived MMP-9 differs from MMP-9 expressed by other cell types in two major ways. First, mature neutrophils do not synthesize MMP-9 *de novo*. Rather, MMP-9 is produced during the late stages of maturation of neutrophils precursors in the bone marrow [27]. These may explain why the numbers of neutrophils were negatively correlated with total MMP-9 level in our study.

There was no significant difference in MMP-9 between 100% oxygen ventilation groups and 45% oxygen ventilation groups. It indicated that FiO<sub>2</sub> was not the regulation factor for the express of MMP-9 in this ventilation animal model. However, the expression of MMP-9 has significant difference in different PIP, in other words, the different mechanical stretch regulated the expression of MMP-9. The application of high pressures to lungs during mechanical ventilation can induce a severe injury type, known as ventilator-induced lung injury [28,29]. Physical stimulus can lead to an inflammatory response within the respiratory system and in distal organs [30]. Several of these pathways result in the synthesis, release, and activation of MMPs [2,31]. Mechanical stretch differentially affects MMP-2/9 and their inhibitors in fetal lung cells [32]. Furthermore, advisable mechanical stretch could promote MMP-9 secretion and decrease the lung injury by our study. The mechanism may be that advisable mechanical stretch activated and enlarged the signal password of MMP-9, leading to MMP-9 synthesis and discharge increase, so the expression of MMP-9 was up-regulated. However, HPIP ventilation formed obvious pulmonary bullae and destroyed the normal pulmonary alveoli structures as well as interrupted the signal connection between cell and cell (**Figure 3(a)**). Finally, the signal password of MMP-9 was broken and the MMP-9 could not be synthesized. Over-mechanical stretch of epithelial cells decreased MMP-9 activity and the MMP-9/TIMP-1 ratio by 60% - 70% [32]. Accordingly, lung injury was inevitable. In LPIP ventilation, there were obvious uneven alveolar ventilation and microatelectasis, suggesting that the stimulation signal transmission of mechanical stretch was uneven in pulmonary alveoli and could not active the signal password of MMP-9, because MMP-9 is not produced constitutively, but needs a trigger to be expressed [33]. MMP-9 is synthesized and stored in the granules of neutrophils and eosinophils in the bone marrow, but is secreted from the cells outside of

the bone marrow in an inducible manner [34,35]. Therefore, atelectasis cannot pass the signal to alveolar epithelial cell, macrophages and fibroblasts and cannot induce MMP-9 synthesis. MMP-9 expression decreased in LPIP and caused lung injury.

In conclusion, different PIP and different oxygen concentrations exert simultaneously on newborn lung in newborn rabbits ventilation; only mechanical stretch stimulation affects MMP-9 synthesis. Advisable mechanical stretch can promote MMP-9 expression and has protective role in lung in VILI. HPIP causes barotraumas and LPIP induces atelectrauma.

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