Original Article
Expressions of matrix metalloproteinase 9 and tissue inhibitor of metalloproteinases 1 in lung tissues of neonatal rats with intrauterine infection

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Abstract: Objective: To investigate the changes and mechanisms of matrix metallopeptidase 9 (MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1) in the lung tissues of neonatal rats with intrauterine infection (II). Methods: The pregnant rats were divided into the intrauterine infection group (group LPS) and the control group (group NS). The rats in group LPS were intraperitoneally injected lipopolysaccharide (LPS) to establish the II animal model; the neonatal rats of the two groups were selected on D1, D3, D7, and D14 after birth for the HE staining and radiation alveolar count (RAC); meanwhile, the protein expression and mRNA quantification of MMP-9 and TIMP-1 in the lung tissues were detected. Results: Group LPS showed significantly increased infiltration of alveolar inflammatory cells and decreased RAC than group NS. The protein and mRNA expressions of MMP-9 in group LPS were significantly higher than group NS on D3, D7, and D14, and those expressions in group LPS showed no significant difference. The protein and mRNA expressions of TIMP-1 in group LPS were gradually increased after birth, and showed significant difference with group NS on D3, D7, and D14. Conclusions: The post-II expressions of MMP-9 and TIMP-1 in the lung tissues of the neonatal rats were significantly increased, and its imbalance might play an important role in repairing lung injuries and abnormalities.

Keywords: Intrauterine infection, bronchopulmonary dysplasia, matrix metalloproteinase, neonatal, rat

Introduction

Intrauterine infection (II) refers to the congenital infection in fetus caused by placental vertical transmission when pregnant woman is infected with a pathogen during pregnancy, and it's the main cause of preterm labor as well as an important factor of short-term and long-term adverse outcomes in preterm children. The incidence of II in preterm children with gestational age less than 28 weeks was as high as 90% [1]. The intrauterine inflammation exposure could cause multiple organ damages, among which the developing lungs are the most vulnerable target organ. Clinical and animal models had all confirmed that maternal chorioamnionitis might promote the maturity of fetal lungs, but it could also increase the risk of bronchopulmonary dysplasia (BPD) in preterm children [2, 3]. In 2012, the meta-analysis about amnionitis and BPD also showed significant correlations between them [4].

The significantly improved survival rate in rescuing preterm children is also accompanied by the increasing incidence of BPD [5]. The incidence of BPD in preterm infants whose birth weight less than 1000 g was about 50% [6]. BPD has seriously affected the survival and life quality of preterm children; according to the recent data, the mortality rate of severe BPD was 25%, and the main causes of death were recurrent lower respiratory tract infection, persistent pulmonary hypertension, pulmonary heart disease, or sudden death. The incidence of neurodevelopmental disorder was 2 to 3 times higher than normal children, and the early mortality was high [7]; so far, there is no effective cure. Previously, classic BPD was related with early oxygen toxicity or mechanical lung injury; with the applications of prenatal hormones as well as the improvements of respiratory support, BPD has exhibited huge differences than before. The pathological features of new BPD were alveolar and pulmonary microvascular dysplasia [8]. Based on the

genetic susceptibility, inflammation caused by oxygen toxicity, pressure injury, or infection in developing lung has been found to be central to the pathogenesis of BPD [9, 10]. The pathogenesis of II-caused BPD is still unclear. During normal development or injury repairing process of lung tissues, the degradation and synthesis of extracellular matrix (ECM) are involved in. Matrix metalloproteinases (MMPs) are a group of zinc ions (Zn^{2+})-dependent endopeptidase family, which could specifically degrade extracellular matrix (ECM) and be regulated by tissue inhibitors of metalloproteinases (TIMPs) and cytokines. Much evidence had shown that MMPs were involved in the respiratory tract reconstruction and ECM rebuilding, and they also could facilitate the migration of the relevant cells during inflammations [11]. MMPs play an important role in the ECM remodeling that occurs during lung morphogenesis [12], inflammation [13], repair after injury [14]. As the mark of lung injury and repairing, MMPs had been more studied in the fields of hyperoxia-induced lung injury or mechanical lung injury, etc. [15, 16]. However, the researches related to the expression changes of MMPs in the post-II lung tissues are rarely reported. In this study, the expressions of MMP-9 and TIMP-1 in the lung tissues of neonatal rats with II were monitored, aiming to explore their mechanisms in the lung injuries of neonatal rats with II.

Materials and methods

Animals

A total of 20 clean healthy adult Wister rats [SCXK (Lu) 20090007] (females weighed 220-260 g and males weighed 280-350 g) were purchased from the experimental animals and animal experiments Center (Qingdao). Breeding conditions: 24±1°C, the females and the males were mated with the ratio as 3:1; the vaginal smear was observed under light microscope on the next day of mating, with sperms covering the whole vision recorded as the zero-day of pregnancy; the rats were then fed individually in cages. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Affiliated Hospital of Qingdao University.

Animal model preparation and grouping

The 15 pregnant rats were randomly divided into group LPS and group NS with the ratio as 2:1. The rats with 15-day pregnancy in group LPS (n = 10) were intraperitoneally injected 300 μg/kg LPS (purchased from Shanghai Sangon Biotech Co., Ltd) slowly, and then continued feeding in the cage. The rats with 15-day pregnancy in group NS (n = 5) were intraperitoneally administered 1 ml/kg saline. The rats in the two groups were performed cesarean section on the 21-day pregnancy. One group in group LPS exhibited stillborn fetuses and the average number of births in the rest 9 groups were six fetuses. The five groups in group NS all survived with an average number of births as 10 fetuses. Six neonatal rats in group LPS and group NS were randomly selected on D1, D3, D7, and D14 after birth, respectively, for the specimen sampling.

Specimen sampling

The pregnant rats in the above two groups were decapitated at the above four time points after cesarean section to sample the lung tissues; after washing, the lung tissues were fixed in formalin, followed by paraffin-embedding. The paraffin sections were then performed the HE staining or immunohistochemistry. Meanwhile, fresh lung tissues were stored at -80°C for RT-PCR.

Radial alveolar count (RAC)

RAC is an important indicator of alveolar development, and it refers to the alveolar number contained in the terminal breathing unit. Under light microscope, the specific counting is the alveolar number contained from the center of the respiratory bronchioles to the vertical line of the nearest fibrous septum or pleura; each group was counted 10 samples, and each slice was counted 5 times for the mean value.

Immunohistochemical assay

The immunohistochemistry kits of MMP-9 and TIMP-1 were purchased from Abcam and Santa. The SP immunohistochemistry kit was used to detect the MMP-9 and TIMP-1 immune activities in the lung tissues. Scoring criteria of immunohistochemistry: A: grades of positive cells: 0 point: 0~1%; 1 point, 1~10%; 2 points,

Table 1. Comparison of RAC between the two groups

Group	D1	D3	D7	D14
LPS	2.180 ± 0.15	3.78 ± 0.434*	5.42 ± 0.42*	6.54 ± 0.47*
NS	2.35 ± 0.19	4.16 ± 0.16	6.67 ± 0.30	8.65 ± 0.46

Note: *$P < 0.01$, compared with group NS.

Table 2. Comparison of MMP-9 and TIMP-1 protein expressions between group LPS and group NS

| Group | LPS | | NS | |
	MMP-9	TIMP-1	MMP-9	TIMP-1
D1	1.33 ± 0.516	1.67 ± 0.816	1.33 ± 0.516	1.67 ± 0.816
D3	4.67 ± 1.966*	4.51 ± 2.168*	1.67 ± 0.516	2.17 ± 0.408
D7	5.83 ± 2.229*	5.83 ± 2.229*	2.33 ± 0.516	2.67 ± 0.516
D14	5.50 ± 0.47*	8.00 ± 1.095**	3.33 ± 0.516	3.33 ± 0.516

Note: *$P < 0.05$, **$P < 0.01$, compared with group NS.

Table 3. Comparison of MMP-9 and TIMP-1 mRNA expressions between group LPS and group NS

| Time | LPS | | NS | |
	MMP-9 mRNA	TIMP-1 mRNA	MMP-9 mRNA	TIMP-1 mRNA
D1	1.23 ± 0.06	1.23 ± 0.06	1.02 ± 0.09	1.03 ± 0.04
D3	1.46 ± 0.08**	1.37 ± 0.07**	1.23 ± 0.08	1.21 ± 0.07
D7	1.58 ± 0.18**	1.50 ± 0.08**	1.29 ± 0.04	1.32 ± 0.06
D14	1.59 ± 0.11**	1.70 ± 0.12**	1.35 ± 0.06	1.41 ± 0.08

Note: **$P < 0.01$, compared with group NS.

10%~50%; 3 points, 50~80%; 4 points, 80~100%; B: grades of staining intensity 0, 1, 2, and 3 represented negative, weakly positive, positive, and strongly positive, respectively. Immunohistochemical score (IHS) = A × B.

RT-PCR

The ultrapure RNA Extraction Kit (CWbio. Co., Ltd, Cat #CW0581) was used to extract the total RNA from the tissue sample for the reverse transcription to synthesize cDNA. Primer sequences: MMP-9 (85 bp), upstream: 5'-GCTTTGCTGATGCTTCAGAA-3'; downstream: 5'-GTTTGGAATCGACCCACGTC-3'; TIMP-1 (85 bp), upstream: 5'-GCCGCCTAAGGAACGGAAA-3'; downstream: 5'-GCACACCCCACAGCCAGCAC-3', GAPDH (138 bp), upstream: 5'-TGGAGTCTACT-GGCGTCTT-3'; downstream: 5'-TGTCATATTTCT-CGTGGTTCA-3'. Amplification program: 95°C for 10 min, 95°C for 15 s, 60°C for 60 s, 45 cycles. Primer Screening: after cDNA of each sample was mixed, this mixture was used as a template for 5-fold dilution, and 2 µL of each diluted sample was used as a template

for the amplification using the target gene primers and the internal reference gene primers; meanwhile, the standard curves of the internal reference gene and the target gene were drawn. Simultaneously, the melting curve was also analyzed. The ABI-7500 fluorescence quantitative PCR instrument was used for the relative quantitative analysis of the data using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis: the data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and performed the statistical analysis using SPSS18.0 software; the intergroup comparison used the t test; the comparison among different time points in group LPS used the analysis of variance; the pairwise comparison used the LSD method, with $P < 0.05$ considered as statistically significant difference.

Results

Conditions of pregnant rats and delivery

All the 15 pregnant rats acted and ate normally, and no pregnant rat died. One group in group LPS exhibited stillborn fetuses and the average number of births in the rest 9 groups were six fetuses. The five groups in group NS all survived with an average number of births as 10 fetuses.

General observation of lung specimens in neonatal rats under light microscope

Changes of lung specimens in neonatal rats under light microscope: group NS: the lung surface was pale red with good elasticity and glossiness; the lung tissue structures and bronchial epithelium were complete; the alveoli were evenly spaced, the alveolar cavity was clear, and the lobular structures were clear; the bronchial cavity and alveolar cavity showed no obvious inflammatory cell and exudate. Group LPS: the lung surface exhibited congestion,
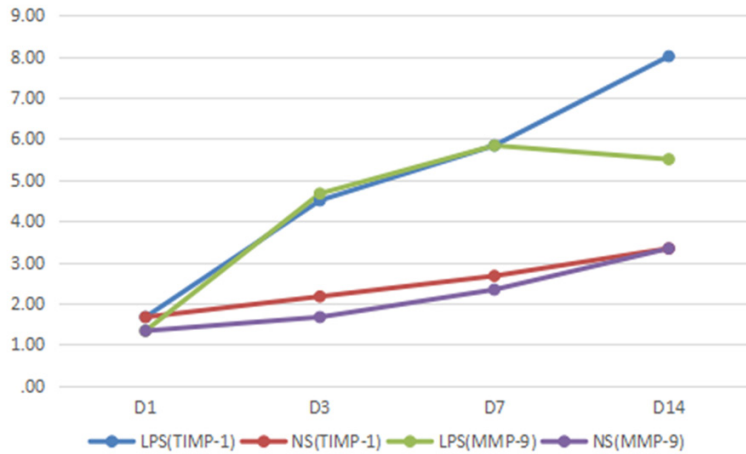
Figure 1. MMP-9 and TIMP-1 protein expressions between group LPS and group NS.

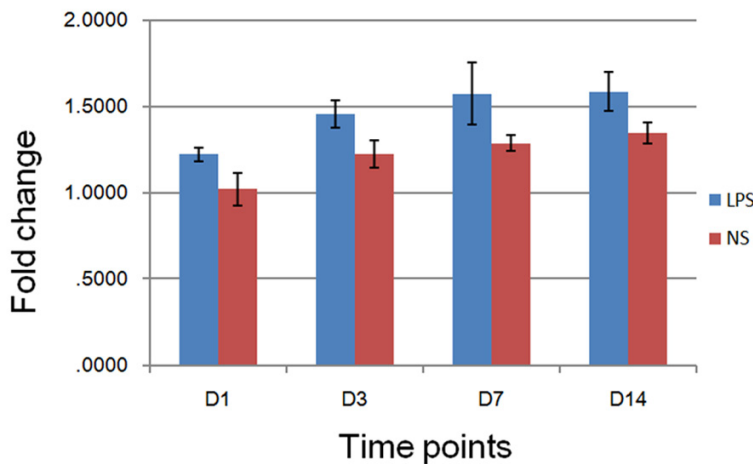**Figure 2.** Expression pattern of MMP-9 mRNA at different time points in LPS and NS groups.

Immunohistochemistry of MMP-9 and TIMP-1 as well as detection of MMP-9 and TIMP-1mRNA

The lung tissues in group NS exhibited none or scattered weakly positive staining of MMP-9 and TIMP-1, and the positive staining was mainly located in partial alveolar epithelial cells of alveolar macrophages; the alveolar basement membrane exhibited weakly positive staining of TIMP-1; group LPS exhibited significantly enhanced expressions of MMP-9 and TIMP-1 than group NS, and the positive expressions were mainly distributed in the airway epithelial cells, basement membrane of alveolar epithelial cells, inflammatory cells, and fibroblasts.

MMP-9 protein expressions and MMP-9 mRNA contents in the two groups: the MMP-9 protein expressions and MMP-9 mRNA contents in group LPS on D3, D7, and D14 were statistically significantly increased than group NS ($P < 0.05$), and the above contents were increased than those on D1 ($P < 0.01$), but there was no statistical difference in the above contents among D3, D7, and D14 (**Tables 2**, **3** and **Figures 1**-**8**).

TIMP-1 Protein Expressions and TIMP-1 mRNA contents in the two groups: the TIMP-1 protein expressions and TIMP-1 mRNA contents in group LPS on D3, D7, and D14 were statistically significantly increased than group NS ($P < 0.01$), which were gradually increased after birth and reached the peaks on D14 (**Tables 2**, **3** and **Figures 1**, **9**, **10**).

Discussion

II is an important cause of short- and long-term adverse outcomes in premature children. The smaller the preterm infants are, the higher the

edema, poor elasticity and glossiness, and spotty or focal bleeding. On D7, partial visions exhibited the widened alveolar walls and spaces; on D14, the alveoli exhibited dysmaturity and were significantly lagged; the number of alveoli was significantly reduced than group NS; the alveolar structures were simple, the volume was increased, and the phenomenon of vesiculation appeared. No significant lung fibrotic change was observed in the both two groups.

Changes of RAC

RACs in group LPS and group NS at all time points were shown in **Table 1**. On D3, D7, and D14, RAC in group LPS was significantly reduced than group NS ($P < 0.05$).
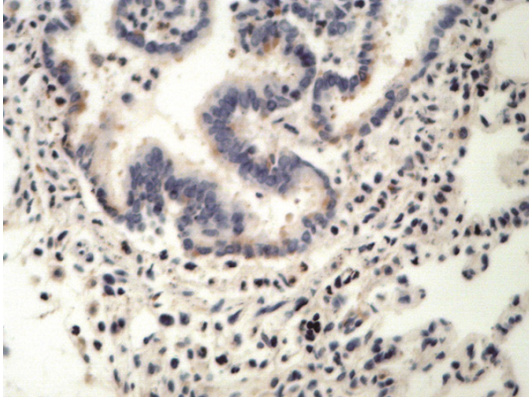
Figure 3. Immunohistochemical results of MMP-9 in the lung tissues of group NS three days after birth (yellow particles indicated the positive result).
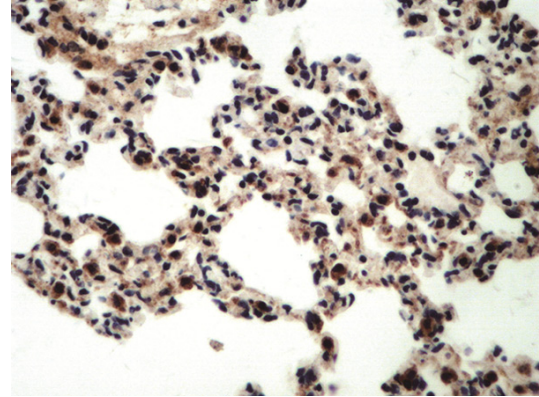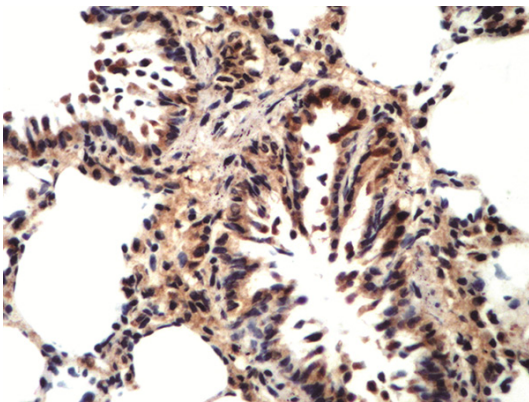

Figure 6. Immunohistochemical results of MMP-9 in the lung tissues of group LPS seven days after birth.

Figure 4. Immunohistochemical results of MMP-9 in the lung tissues of group LPS three days after birth (yellow particles indicated the positive result).
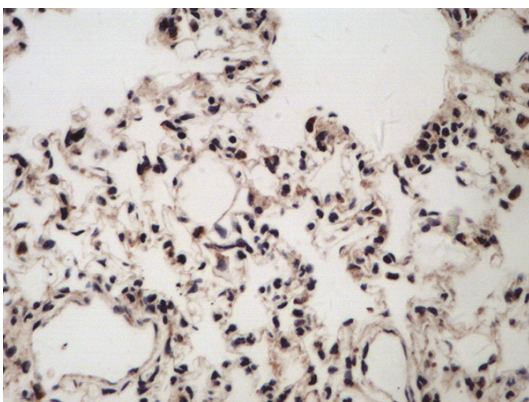

Figure 5. Immunohistochemical results of MMP-9 in the lung tissues of group NS seven days after birth.

II incidence will be. Although the survival rate of premature children has been significantly improved in recent years, the incidence of BPD has also been increased year by year. BPD is one common serious adverse outcome of the respiratory system in extremely premature infants, and it has become one of the most intractable problems of neonatal intensive care unit (NICU). In normal lung tissues, the synthesis and degradation of ECM maintain homeostasis; ECM could maintain the alveolar structures, which is very important to ensure the normal lung functions. The pathological change of BPD is alveolar hypoplasia and altered microvascular maturation. MMPs could degrade the basement membrane of alveolar epithelial cells and participate in the conversion and replacement of the basement membrane, thus playing an important role in the occurrence and development of BPD. This study was designed to study the mechanisms of MMPS in repairing post-II lung tissue injuries.

Similar to the developments of human lung, the rat lung development could also be divided into the embryonic stage, pseudoglandular stage, canalicular stage, saccular stage, and alveolar stage [17]. At birth, the rat lung would still be in the saccular stage, and the alveoli have not developed yet; therefore, neonatal rats are suitable for studying the premature lung development. In this study, the II model was established through intraperitoneal injection of LPS, and it was found that group LPS exhibited reduced natural delivery numbers and alive neonatal rats than group NS, which was considered to be related with II.

The two weeks after birth is a critical period in rat alveolar development, and RAC could reflect the alveolar number contained in the terminal
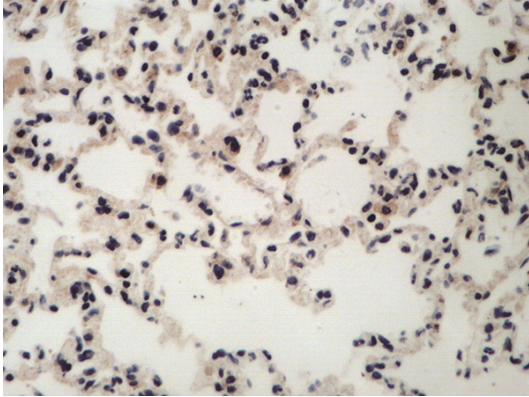
Figure 7. Immunohistochemical results of MMP-9 in the lung tissues of group NS 14 days after birth.
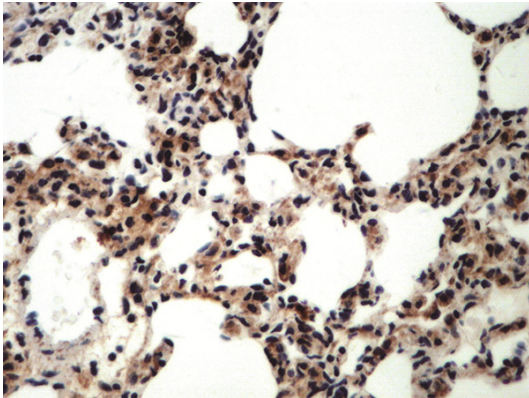

Figure 8. Immunohistochemical results of MMP-9 in the lung tissues of group LPS 14 days after birth.
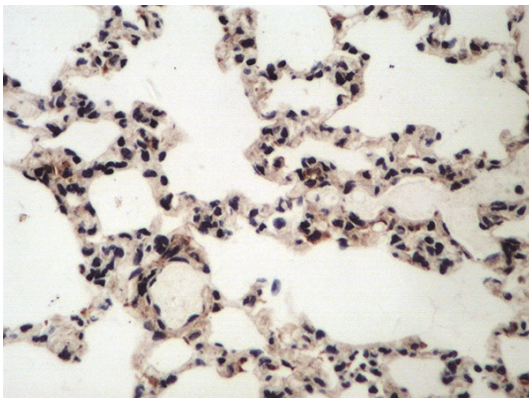

Figure 9. Immunohistochemical results of TIMP-1 in the lung tissues of group NS seven days after birth.

Figure 10. Immunohistochemical results of TIMP-1 in the lung tissues of group LPS seven days after birth.

respiratory units, so it is an important parameter to evaluate the alveolization process and lung development maturity. Fro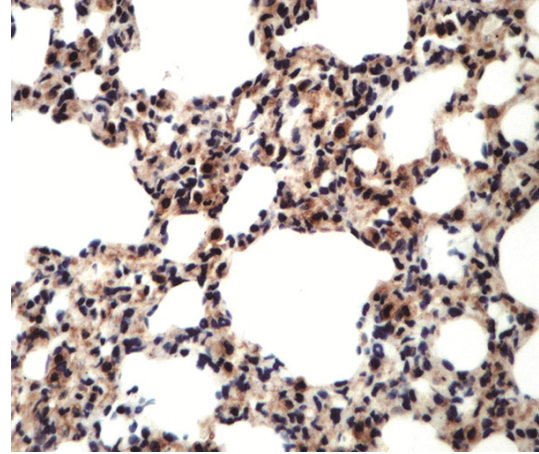m the pathological changes of lung tissues and RAC in this study, it could be concluded that the rat lung tissues exhibited inflammatory infiltration after II, and the number of alveoli was reduced on D7 and D14; partial alveolar volumes were increased, and the alveolar septum was thickened, so it confirmed that II could lead to lung injury, and the pathological characteristics was similar to BPD.

Previous research found the expressions or activities of MMP-2 and MMP-9 were increased in the bronchoalveolar lavage fluid in LPS, ischemia-reperfusion, high oxygen and mechanical ventilation induced lung injuries, and the mRNA and protein expressions and activities of MMP-2 and MMP-9 in lung tissues were upregulated [18]. This study found that the expressions of MMP-9 and TIMP-1 in rat lung tissues on D3, D7 and D14 were increased than the control group, prompting that MMP-9 and TIMP-1 were involved in the repairing process of II-induced lung injuries. The main function of MMPs is to degrade ECM, which could not only provide the connecting and supporting roles among cells but also control the differentiation, adhesion, proliferation, and migration of cells, thus impacting the survival and growth of cells. The maturation of alveolar walls and pulmonary vascular walls depend on the changes of ECM. The main constituent of the alveolar basement membrane is type IV collagen, whose main degrading enzymes are MMP-9 and MMP-2. TIMPs is a specific inhibitor of MMPs. The homeostasis between these two is the first condition to ensure the integrity of ECM. It was confirmed that during the updating process of

lung matrixes, MMPs is one of the important parts, promotes the angiogenesis, and makes the inflammatory cells to migrate towards the infected or damaged lung tissues, thus generating immune protections [19]. The metabolic disorders of ECM (including the alveolar basement membrane) might have a key role in the formation of BPD, and the alveolar basement membrane injury, which has the type IV collagen as the main component, is a key event towards the pathological changes of BPD. Among the chronic lung diseases in preterm children, one or more MMPs might be upregulated [20, 21], and the more severe the injury, the stronger the expression level; these two exhibited certain positive correlation [22], consistent with this study.

MMPS are mainly produced by macrophages and have regulatory effects towards the activities of a variety of chemokines and growth factors [23]. Growth factors, transcription factors, and cytokines are also the regulatory factors of MMPs, and other factors such as environmental and mechanical factors, endogenous inflammatory mediators, or pathogens also have regulatory effects. These factors are not only the regulatory factors of MMPS but also the substrates, and they would interact with each other to ensure the effective regulation and activation of MMPS. The occurrence sign of BPD is continuous inflammations on the cellular level; II would lead to the accumulation of the inflammatory cells inside the alveoli; after that, a large number of inflammatory cytokines would be released, MMP-9 would be upregulated, ECM's degradation would be more active, and TIMP-1 would also be compensatorily upregulated. After MMPS is activated, the lung basement membrane is degraded and injured, leading to further infiltration of inflammatory cells and activation-caused waterfall of inflammatory cytokines, which would further aggravate the lung injury, interfere the normal replacement of alveolar type II cells, cause the production of pulmonary surfactant to be decreased; meanwhile, it could also promote the migration of fibroblasts and the deposition of interstitial collagens among alveolar spaces. This study found that MMP-9 showed no significant expression difference on D3, D7, and D14 after II, but TIMP-1 was gradually upregulated with the age increasing and reached the peak on D14, indicating that although MMP-9 and TIMP-1 in lung

tissues were upregulated after II, they did not exhibit synchrony, and the imbalance of MMP-9/TIMP-1 might be the mechanism of II-resulted impaired alveolarisation and abnormal repairing of lung tissues. The end-point outcome of BPD is the excessive ECM deposition caused pulmonary fibrosis, which is the result of the synthesis-degradation imbalance of ECM. During the ECM remodeling phase, MMPs are mainly produced by the fibroblast-based stromal cells, and are mainly from inflammatory cells in the early stage. In addition to ECM which mainly produce collagens, the fibroblasts also generate MMPs and TIMPs simultaneously. In the pathogenesis of acute lung injury, MMPs inhibitors played important protective roles in the early stage of inflammation [24]. Study had also found that the ratio of MMP-9/TIMP-1 had critical roles in the development process of ARDS: if MMP-9/TIMP-1 was < 1, it meant the possibility of pulmonary fibrosis after lung injury [25]. The semiquantitative analysis of BPD's clinical data revealed that the staining intensities of MMP-1 and TIMP-2 in alveolar type II cells showed no difference in various stages of BPD, but in the chronic phase, the intensity of TIMP-1 was significantly enhanced than the previous stage. This relative increasing of TIMP-1 indicated that the collagen degradation was weakened in the chronic stage of BPD [26]. The limitation of this study was that the levels of cytokines such as IL-6 and IL-8 in amniotic fluid or alveolar lavage fluid were not simultaneously detected for the evaluation of the severity of intrauterine infection. Because MMP plays important roles in regulating the occurrence and remodeling of angiogenesis and vascular endothelial growth factor (VEGF) was an important cytokine in regulating the development of lung vessels. Studies in future could be focused on simultaneously detecting the expression of VEGF in lung tissues to explore the correlations between MMP and VEGF in intrauterine infection-associated lung injury. The changes of these cytokines as well as their correlations with MMP/TIMP might be helpful to better interpret the pathogenesis of intrauterine infection-associated lung injury.

This study dynamically monitored the expressions of MMPs/TIMPs in the lung tissues of premature neonatal rats with II, confirmed their participation in the process of lung injury and repairing, initially described its mechanisms in

the post-II lung injuries. Therefore, dynamically monitoring the changes of MMPs and TIMPs expressions in II-caused lung injuries could help to early determine the occurrence and outcome of BPD; thorough studying the changing rules of MMPs/TIMPs could help the early prediction of BPD, or through regulating the appropriate expression of MMPs/TIMPs to reduce lung injuries so as to bring new perspectives and directions for the prevention and treatment of BPD.

Immunohistochemical results of MMP-9 in lung tissues (light microscopy ×400): the yellow granules indicated positive result, the more the yellow particles, the stronger the staining is, the more expressions of the protein will be. On D3 and D7, the MMP-9 staining in group LPS (**Figures 4**, **6**) was significantly enhanced than group NS (**Figures 3**, **5**). On D14, the MMP-9 staining in group LPS (**Figure 8**) was significantly enhanced than group NS (**Figure 7**).

Immunohistochemical results of TIMP-1 in lung tissues (light microscopy ×400): the yellow granules indicated positive result, the more the yellow particles, the stronger the staining is, the more expressions of the protein will be. On D7, the TIMP-1 staining in group LPS (**Figure 10**) was significantly enhanced than group NS (**Figure 9**).

Disclosure of conflict of interest

None.

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