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# Expression of transforming growth factor-$\beta_1$ mRNA and protein in premature rats with chronic lung disease

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**Abstract: Objective** To investigate the dynamic changes of transforming growth factor-$\beta_1$ (TGF-$\beta_1$) mRNA and protein expression and their effects on lung development in premature rats with chronic lung disease (CLD). **Methods** CLD was induced by hyperoxia exposure ($FiO_2$ = 0.90) in neonatal premature rats. The dynamic changes of lung coefficient and radical alveolar counts (RAC) were observed; and the expressions of TGF-$\beta_1$ mRNA and protein were assayed with reverse transcription polymerase chain reaction (RT-PCR), immunohistochemical and image processing technique on days 1, 3, 7, 14 and 21 after birth. For comparison analysis, 30 premature rats exposed to air were used as the Control group. **Results** There were no differences in the lung coefficient and the RAC between the CLD and the Control groups within 3 days after birth. However on the 7th and 14th days, the lung coefficient and the RAC of the CLD group were significantly lower than those of the Control group. The RAC decreased to the nadir on the 21st day, but the lung coefficient was not different from the Control group. In the CLD group, the expression level of TGF-$\beta_1$ mRNA increased on the 3rd day, peaked on the 14th day and remained higher on the 21st day; the higher expression of TGF-$\beta_1$ protein was detected on the 7th day and peaked on the 21st day; and the TGF-$\beta_1$ protein expression was negatively correlated with RAC($P$ = 0.0027). **Conclusions** The over-expression of TGF-$\beta_1$ in lung tissues is associated with the lung development disorder. The TGF-$\beta_1$ expression level may be an index for the assessment of lung development.

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**Key words:** Transforming growth factor-$\beta_1$; Chronic lung disease; Rat, premature

## 慢性肺疾病早产鼠肺组织转化生长因子 $\beta_1$ 基因及蛋白表达的动态变化

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[**摘 要**] **目的** 近年来研究发现慢性肺疾病(CLD)早产儿支气管肺泡灌洗液中转化生长因子 $\beta_1$(TGF-$\beta_1$)水平明显升高,但其表达在 CLD 发生、发展中动态变化规律及与 CLD 的肺泡发育障碍的关系尚不明确。该研究探索高氧致 CLD 肺组织 TGF-$\beta_1$ 基因及蛋白表达特点及对其肺发育的影响。**方法** 采用高浓度氧诱导早产鼠 CLD 模型,应用反转录聚合酶链反应(RT-PCR)、免疫组织化学及图像分析等技术,动态观察肺系数、放射状肺泡计数(RAC)的变化,并同时测定肺组织 TGF-$\beta_1$ mRNA 及蛋白表达水平。**结果** 生后 1 d,3 d,两组肺系数、RAC 无差异($P$>0.05),7 d 和 14 d 时实验组肺系数、RAC 低于对照组($P$<0.05),21 d 时 RAC 明显降低($P$ <0.001),但两组肺系数无差异;实验组肺组织 TGF-$\beta_1$ mRNA 水平 3 d 高于对照组($P$ =0.005),14 d 达高峰($P$ <0.001),21 d 稍有下降,但仍高于对照组($P$ =0.005);实验组肺组织 TGF-$\beta_1$ 蛋白表达 7 d 增高($P$=0.036),21 d 达高峰($P$ < 0.001);肺组织 TGF-$\beta_1$ 蛋白表达与 RAC 呈显著负相关($P$ = 0.003)。**结论** 暴露高氧环境中早产鼠肺组织 TGF-$\beta_1$ 蛋白表达的动态变化与其肺发育障碍的程度相一致,TGF-$\beta_1$ 是抑制肺泡发育的重要因子。

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[**关 键 词**] 转化生长因子 $\beta_1$;慢性肺疾病;早产鼠

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In recent years, with the increase of survival rate of very low birth weight (VLBW) and extremely low birth weight (ELBW) neonates, the incidence of chronic lung disease (CLD) of prematurity increased

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progressively. In previous studies, it was thought that pulmonary interstitial fibrosis was a final pathological change of CLD, whereas recent studies have indicated that the lung development disorder is the main characteristic of CLD. Transforming growth factor $\beta_1$ (TGF-$\beta_1$) is an important growth regulation factor, which plays an important role in the regulation of growth, differentiation, and repair in a wide variety of cells and tissues. However, the TGF-$\beta_1$ gene and protein expression changes and their effects on lung development in the pathogenesis and development of CLD have not been fully identified. This study aimed at investigating the changes of TGF-$\beta_1$ in the pathogenesis and progression of CLD and its effect on postnatal development of alveoli in preterm infants.

## Material and methods

### Material

Sprague-Dawley (SD) rats (on the 21st day of gestation) were purchased from Animal Center of the China Medical University. Rabbit anti-rat TGF-$\beta_1$ antibody, SABC and the DAB kit were purchased from Boster (Wuhan, China). Trizol reagent was purchased from Promega. The RT-PCR kit was purchased from Takara. PCR primers for TGF-$\beta_1$ and internal control were synthesized by Aoke Bio-engineering Company (Beijing, China).

## Methods

### Animals and grouping

Cesarean section was performed on the 21st day of gestation. Neonatal premature rats without gender limitation were randomly assigned into a Control group ($FiO_2$ 0.21, $n$ = 30) and a CLD group ($FiO_2$ 0.90, $n$ = 30) according to $FiO_2$. The mean body weights of the Control group and the CLD group were 4.83 ± 0.12 g and 4.84 ± 0.13 g, respectively.

### Animal CLD

In the CLD group, premature SD rats were put into an oxygen chamber immediately after birth. Oxygen was continuously supplied at a $FiO_2$ of 0.90 and $CO_2$ concentration <0.5% ($CO_2$ was absorbed by natrica calx). The chamber temperature was maintained at between 25-27℃, and relative humidity, between 50%-70%. The chamber was opened for 1 hour each day to add water, food and to exchange padding. The rat's foster mothers were exchanged with the Control group in order to avoid the decrease of feeding ability caused by oxygen intoxication. The $FiO_2$ of the Control group was 0.21 (air), and the detail of the method and experimental control factors were similar to the CLD group[1].

### Collection of samples

Six rats were randomly selected and sacrificed at the 1st, 3rd, 7th, 14th and 21st days in both group. The left lung was taken out, weighed and fixed in 4% paraformaldehyde, and then embedded in paraffin. Sections (5μm) were prepared for the observation of histological changes (light microscope) and immunohistochemistry. The right lung was removed and placed into Eppendorf tubes, and stored in a -80℃ refrigerator for RT-PCR detection.

### Lung coefficient

Lung coefficient refers to the ratio of lung weight (mg) to body weight (g), which can reflect the stage of lung development directly and the severity of pulmonary interstitial fibrosis indirectly.

### Radical alveolar counts (RAC)

RAC refers to the number of alveoli transected by a perpendicular line drawn from the center of the most peripheral bronchiole (recognizable by not being completely covered by epithelium) to the pleura or the nearest interlobular septum. This is an important index to evaluate the stage of lung development. Five hematoxylin & eosin staining sections of each time point in each rat were randomly selected. Five fields for each section were examined under a light microscope (×100) in order to estimate RAC and calculate the mean value[2].

### TGF-$\beta_1$ protein expression

TGF-$\beta_1$ protein expression was measured by SABC immunohistochemistry method. PBS was used to replace anti-rat TGF-$\beta_1$ antibody for negative control. TGF-$\beta_1$ positive cells were indicated by the presence of brown particles in the cytoplasm of cells. Five TGF-$\beta_1$ immunohistochemistry staining sections of each time point in each rat were randomly selected, and 5 fields for each section were examined under a light microscope (×400). Universal imaging corporation image analysis system (USA) and Meta Morph software were used to measure the mean gray scale value (MGSV) which indicated the intensity of staining. The lower the MGSV, the higher the positive expression, which indicated a high protein content.

### TGF-$\beta_1$ mRNA expression

TGF-$\beta_1$ mRNA expression was measured by RT-PCR. Total RNA was extracted using Trizol. cDNA was synthesized using RT kit and then amplified by

PCR. Primers sequences were as follows: 1) TGF-$\beta_1$ upstream: 5′-AGG CGG TGC TCG CTTTGT-3′, and downstream: 5′-TCC CGA ATG TCT GACGTATTGA-3 (202bp); 2) β-actin (internal control) upstream: 5′-GAT TGC CTC AGG ACA TTT CTG-3′, and downstream: 5′-GAT TGC TCA GGA CAT TTC TG-3′ (690 bp). The reaction condition of PCR was as follows: denaturation at 94℃ for 3 minutes, 94℃ for 45 seconds, and 58℃ for 1 minute, for 35 cycles, then elongation at 72℃ for 7 minutes. Relative mRNA level was expressed as the absorbance ratio of TGF-$\beta_1$ and β-actin.

**Statistical analysis**

Statistical analysis was performed using SPSS 10.0 software. All data were presented as $\bar{x} \pm s$. The Dunnet-$t$ test was applied for inter-group comparison, and correlation was evaluated with Spearman analysis.

## Results

**Morphological changes of lung tissues**

On the 1st day after birth, the irregular structure of pulmonary alveoli with small alveoli lumen and thick alveoli septum was observed in each group. On the 3rd day, alveoli structure became regular, the septum became thinner and small amount of inflammatory cells infiltration in the alveoli and septum was observed in the CLD group. Between 7 and 21 days, alveoli were regular in size in the Control group. However in the CLD group, the alveoli enlarged on the 7th day along with alveoli wall thinning, and on the 14th day alveoli enlarged more significantly and the number of alveoli decreased; on the 21st day normal alveoli structure disappeared, the number of alveoli decreased more significantly, the alveolar diameter increased apparently and the number of local pulmonary interstitial cell increased (Figure 1).

**Lung coefficient**

No difference was observed in the lung coefficient between the two groups within 3 days after birth. However on the 7th and 14th days, the lung coefficient of the CLD group was significantly lower than that of the Control group. The difference was not found on the 21st day (Table 1).

**Table 1 Lung coefficient in the CLD and Control groups**

($n=6, \bar{x} \pm s$, mg/g)

| Group | 1 d | 3 d | 7 d | 14 d | 21 d |
|---|---|---|---|---|---|
| Control | 5.12 ± 0.22 | 6.23 ± 0.54 | 8.04 ± 0.60 | 5.86 ± 0.27 | 3.81 ± 0.45 |
| CLD | 5.08 ± 0.16 | 6.12 ± 0.49 | 6.08 ± 0.65 | 4.54 ± 0.36 | 3.90 ± 0.34 |
| $t$ | 0.58 | 0.56 | 4.53 | 3.91 | 0.67 |
| $P$ | >0.05 | >0.05 | 0.004 | 0.007 | >0.05 |

**RAC**

There was no difference in the RAC between 2 groups within 3 days after birth. However on the 7th and 14th days, the RAC of the CLD group was significantly lower than that of the Control group, and the difference peaked on the 21st day (Table 2).

**Table 2 RAC in the CLD and Control groups**

($n=6, \bar{x} \pm s$)

| Group | 1 d | 3 d | 7 d | 14 d | 21 d |
|---|---|---|---|---|---|
| Control | 4.83 ± 0.68 | 6.17 ± 0.71 | 7.67 ± 0.52 | 9.03 ± 0.74 | 9.83 ± 0.65 |
| CLD | 4.75 ± 0.79 | 6.23 ± 0.91 | 6.35 ± 0.83 | 5.15 ± 0.75 | 4.72 ± 0.31 |
| $t$ | 0.63 | 0.48 | 4.00 | 5.21 | 9.61 |
| $P$ | >0.05 | >0.05 | 0.043 | 0.002 | <0.001 |

**TGF-$\beta_1$ protein expression**

In the Control group (day 1- day 21), weak TGF-$\beta_1$ protein expression was only seen in the bronchial epithelium or vessel endothelium and surrounding connective tissues. In the CLD group, the TGF-$\beta_1$ protein expression was not different from the Control group on the 1st and 3rd days. However on the 7th day, higher expression of TGF-$\beta_1$ protein was detected in the alveoli macrophage, epithelium and pulmonary interstitial cells. The number of positive cells increased significantly on the 14th day. The TGF-$\beta_1$ protein was expressed in a lot of alveoli epithelium and interstitial cells in the CLD group on the 21st day (Table 3 and Figure 2).

**Table 3 TGF-$\beta_1$ protein expression in the CLD and Control groups**

($n=6, \bar{x} \pm s$)

| Group | 1 d | 3 d | 7 d | 14 d | 21 d |
|---|---|---|---|---|---|
| Control | 85.24 ± 5.65 | 86.17 ± 3.99 | 84.56 ± 2.14 | 84.56 ± 2.56 | 84.16 ± 2.16 |
| CLD | 86.82 ± 6.80 | 85.92 ± 5.53 | 80.89 ± 1.36 | 73.83 ± 5.21 | 72.99 ± 5.00 |
| $t$ | 0.41 | 0.32 | 2.15 | 5.63 | 4.96 |
| $P$ | >0.05 | >0.05 | 0.036 | 0.003 | 0.004 |

**TGF-$\beta_1$ mRNA expression**

No difference was observed in the TGF-$\beta_1$ mRNA expression between the two groups on the 1st day after birth. However on the 3rd and 7th days the expression level of the CLD group was significantly higher than that of the Control group, and peaked on the 14th day, then decreased somewhat on the 21st day but was still higher than that of the Control group (Table 4).

**Correlation between TGF-$\beta_1$ expression and RAC**

The TGF-$\beta_1$ protein expression level was negatively correlated with RAC in the CLD group ($r=-0.703$, $P=0.0027$).

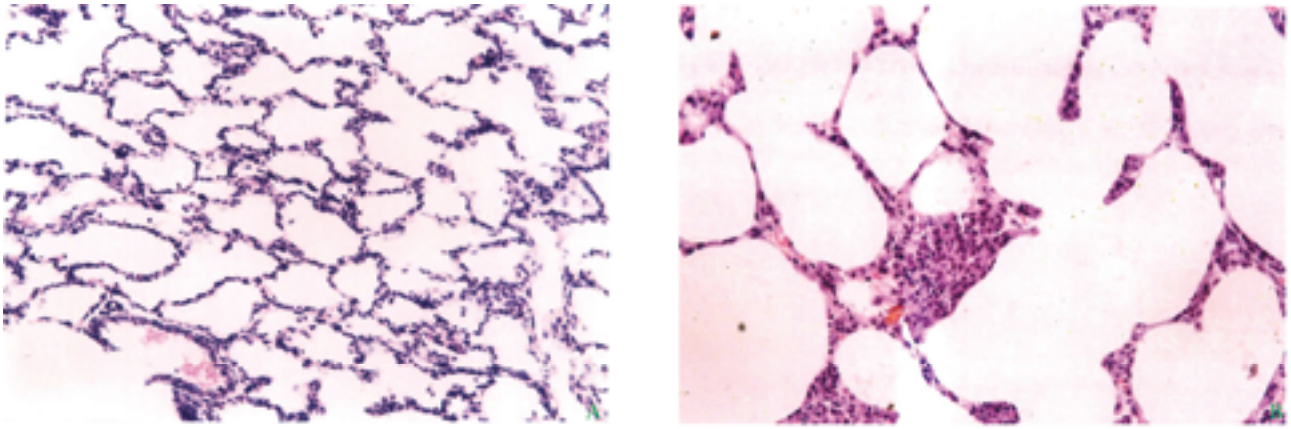

**Figure 1 Morphological changes in lung tissue on the 21st day** (Haematoxylin & Eosin ×200) A: Control group; B: CLD group

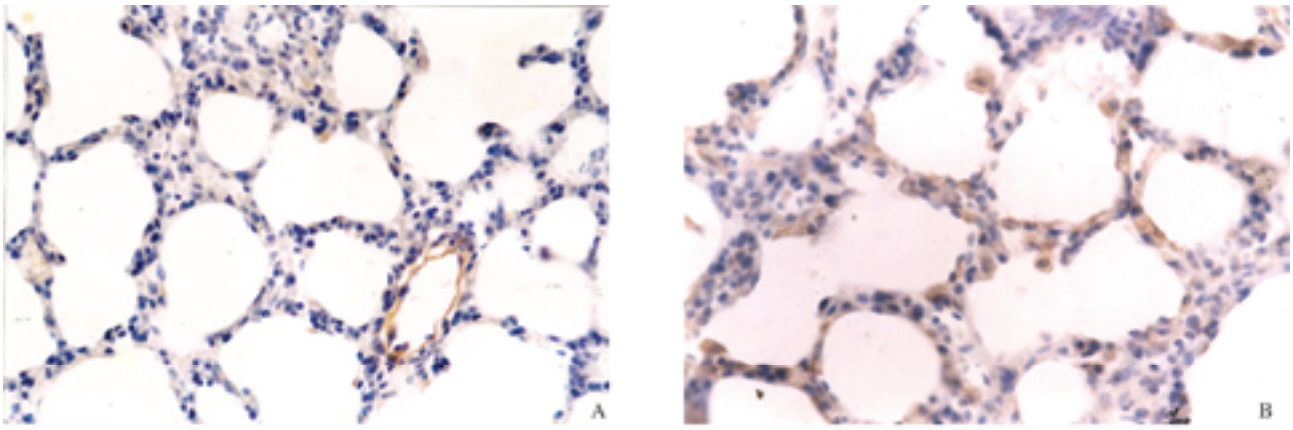

**Figure 2 TGF-$\beta_1$ protein expression in lung tissue on the 21st day** (SABC × 400) A: Control group; B: CLD group

**Table 4 TGF-$\beta_1$ mRNA expression in the CLD and Control groups** ($n=6, \bar{x} \pm s$)

| Group | 1 d | 3 d | 7 d | 14 d | 21 d |
|---|---|---|---|---|---|
| Control | 1.04 ± 0.10 | 1.05 ± 0.12 | 1.03 ± 0.07 | 1.07 ± 0.08 | 1.06 ± 0.09 |
| CLD | 1.03 ± 0.09 | 1.20 ± 0.08 | 1.24 ± 0.15 | 1.38 ± 0.11 | 1.24 ± 0.05 |
| $t$ | 0.23 | 2.87 | 3.62 | 7.12 | 3.37 |
| $P$ | >0.05 | 0.031 | 0.005 | <0.001 | 0.005 |

## Discussion

Recent studies have indicated that alveoli development disorder is the main pathological changes of CLD in VLBW neonates, whereas pulmonary fibrosis is relative mild. Based on these findings, it was speculated that these changes may have resulted from the inhibition of lung tissue development from vesicle to alveoli[3]. The studies have suggested that the retardation of lung development is the new characteristic of CLD, and that these kinds of pathological changes are the results of interactions of high concentration $O_2$, mechanical ventilation pressure and infection. Moreover, the initiation time, trend and change pattern of lung development are difficult to clarify because of the limited number of autopsy cases.

In this study, SD rats with 21 days of gestation were taken out by cesarean section and were put into an environment of high concentration oxygen immediately after birth till the 21st day. The CLD model is in accordance with the pathogenesis and development process of CLD[4,5]. The result indicated that high concentration oxygen exposure for only 7 days caused lung development disorder. With the elongation of high oxygen exposure time, the lung index and RAC decreased progressively, which indicated that the alveoli development disorder worsened. But no difference was observed on the 21st day. It was speculated that this finding was related to pulmonary interstitial cell proliferation and collagen deposition[9] as well as poor nutrition status such as body weight loss. Therefore the deterioration of alveoli development is the main pathological character of lung injury induced by high concentration oxygen in premature rats, which finally results in a greater alveoli diameter and effective ventilation area reduction. These changes affect the air exchange process of lung tissues significantly.

A lot of studies have indicated that the overexpression of TGF-$\beta_1$ is related to the pathogenesis of adult idiopathic pulmonary interstitial fibrosis and

bleomycin induced rat pulmonary fibrosis. O′Reilly[7] reported that in adult rats exposed to a high oxygen environment, the expression of TGF-$\beta_1$ in bronchial epithelium cells and alveoli endothelium cells began to increase at 3 hours after exposure, and reached a peak at 48 hours, then decreased to normal level at 72 hours. Josson[8] reported that in CLD neonates 1 week after birth, the TGF-$\beta_1$ level in bronchoalveolar lavage (BALF) was significantly higher than that of respiratory difficult syndrome (RDS) neonates or neonates without pulmonary disease, and maintained a high level to the 2nd week even the 4th week. Autopsy also indicated positive expression of TGF-$\beta_1$ in the CLD neonatal lung[8]. Therefore it was speculated that the pathogenesis of CLD is closely related to TGF-$\beta_1$. Previous study by the authors indicated that TGF-$\beta_1$ over-expression was closely related to extracellular matrix (ECM) accumulation in pulmonary tissues in premature rats with CLD induced by high concentration oxygen exposure[9]. This study indicated that in premature rats exposed to a high oxygen environment the TGF-$\beta_1$ protein expression peak in pulmonary tissues was a little later than gene expression, but the changing trend was similar. This result was similar to the TGF-$\beta_1$ protein and gene expression pattern reported in bleomycin induced rat pulmonary fibrosis CLD. When comparing with the adult rats exposed to high oxygen, the expression site of TGF-$\beta_1$ in the lung was similar but the duration was significantly longer.

Currently, the study of TGF-$\beta_1$ function mechanism is mainly focused on the effect on ECM such as collagen synthesis. It has been reported that the TGF-$\beta_1$ level in the BALF of 2-3 days old neonates is negatively related to the gestational age, therefore some researchers have suggested using TGF-$\beta_1$ level in BALF (24 hours after birth) to evaluate the long-term prognosis of lung tissues in preterm infants[10]. But this suggestion was based only on clinical observation and lacks related experimental basis. There has not been any research on the relationship between TGF-$\beta_1$ expression in CLD lung tissues and lung development disorder. This study indicated that 7 days after exposure to high concentration oxygen the TGF-$\beta_1$ protein expression level in lung tissues of premature rats was significantly negatively correlated with the RAC index, suggesting that the over-expression of TGF-$\beta_1$ in lung tissues was closely related to the alveoli development disorder. These results supported the suggestion of using neonatal BALF TGF-$\beta_1$ level as an index to evaluate the lung development. But the mechanism of TGF-$\beta_1$ in inhibiting lung development is still unclear. The study in vitro indicated that TGF-$\beta_1$ could induce apoptosis of pulmonary epithelium cells[8]. Buckley[11] measured TGF-β peptide production by AEC2 and macrophages from lungs of adult male rats exposed to 100% oxygen for 48 hours and then allowed to recover in room air for 72 hours. The results showed that the nadir of active TGF-β production by AEC2 coincided with the peak of the AEC2 proliferative phase of repair. Based on these findings, it was speculated that TGF-$\beta_1$ could promote the apoptosis of pulmonary epithelium and inhibit the proliferation and then interfere the lung development, but the exact mechanism needs further studies.

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