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Prevention of Pulmonary Fibrosis via Trichostatin A (TSA) in Bleomycin Induced Rats

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Abstract. *Purpose:* To investigate the effects of non selective histone deacetylase inhibitors Trichostatin A (TSA)on bleomycin-induced pulmonary fibrosis. To investigate the effects of non selective histone deacetylase inhibitors Trichostatin A (TSA) on $HDAC_2$, p-SMAD2, $HDAC_2$ mRNA, SMAD2mRNA in pulmonary fibrosis rats and investigate impossible mechanism. *Methods:* 46 SPF level male SD rats were randomly divided into four groups: ten for normal control group, fourteen for model control group I, twelve for model control group II and ten for treatment group. Rat pulmonary fibrosis was induced by bleomycin(5mg/kg) via single intratracheal perfusion in the two model control groups and treatment group. Normal control mice were instilled with a corresponding volume of 0.9% saline intratracheally. Treatment group was treated by the dilution of TSA 2mg/kg DMSO 60ul and0.9% saline 1.2ml intraperitoneal injection from the next day ,once a day for three days. Model control group II was treated by the dilution of DMSO 60ul and0.9% saline 1.2ml intraperitoneal injection from the next day once a day for three days. Model control group I and normal control group were treated by 0.9% saline 1.2ml intraperitoneal injection from the next day once a day for three days. All the animals were sacrificed on the 21 day after modeling. The pathological changes were observed by hematoxylin and eosin(HE)stain and masson trichrome stain. The expression of $HDAC_2$ mRNA,SMAD2 mRNA were measured by real-time PCR. The protein level of $HDAC_2$ and p-SMAD2 in serum was measured by Western blot. *Results:* The pulmonary fibrosis in treatment group were significantly alleviated compared to the two model control groups ($P<0.05$). Real-time PCR showed that the treatment group had lower expression of lung tissue $HDAC_2$ mRNA than the two model control groups and normal control group ($P<0.05$). The expression of lung tissue SMAD2 mRNA increased in the two model control groups and treatment group ($P<0.05$),but there were no significant differences among the three groups($P>0.05$). Western blot indicated that the protein level of $HDAC_2$ and p-SMAD2 in serum increased in the two model control groups compared with normal control group($P<0.05$).But treatment group had lower protein level of $HDAC_2$ ($P<0.05$) and no significant difference in the protein level of p-SMAD2 compared to the two model control groups ($P>0.05$). *Conclusion:* Non selective histone deacetylase inhibitors of Trichostatin A (TSA) can reduce the bleomycin induced pulmonary fibrosis in rats. TSA attenuates pulmonary fibrosis and it can inhibit $HDAC_2$ expression at the gene and protein level. Bleomycin induced fibrosis has the relationship with p-SMAD2 in gene and protein levels, but TSA inhibit bleomycin-induced lung fibrosis effect with no relation with SMAD2 phosphorylation pathways. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 211-214)*

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Pulmonary fibrosis generally refers to lung inflammation induced by stimulation of different factors of spontaneous immune responses, poison, adverse drug reaction, infection, severe trauma to injury. It reflects as the main feature of alveolar surface epithelial cells injury, inflammatory cells accumulation, fibroblast proliferation, extracellular matrix deposition and scar formed. The final result is normal lung tissue structure change and loss of function which is the end result of interstitial lung disease. More than 5 millions of people in the world suffer from pulmonary fibrosis of different degree (1. In recent years, the incidence rate of pulmonary fibrosis disease caused by various reasons increases obviously, the treatment is extremely difficult, the traditional glucocorticoid has efficacy little and side effects of larger, so it is necessary to explore new therapeutic method.

Histones are important components of the nucleus of the eukaryotic chromosome. N tail of core histones can have effect through various modified with DNA and other protein with the adjustment the nucleosome and chromatin structure and so that the repressed genes restored the activity (2). Histone acetylation /deacetylation modification is one of the key mechanisms of gene transcription regulation with these modifications by histone acetyltransferases HAT and histone deacetylase HDACs (3). HAT urges chromosome depolymerization with activation of transcription; while HDACs closed DNA with inhibiting transcription process. Several recent studies show that HAT / HDACs balance disorder will not only make the gene expression out of control and leading to the tumors occurrence (4), but also related to some disease with the main characteristic of chronic fibrosis (5), such as idiopathic pulmonary fibrosis, chronic kidney disease, heart failure, systemic sclerosis. So the histone deacetylase HDACs and its inhibitor HDACi received more and more attention in the research of etiology and treatment of these diseases. New hope has probably been brought by the histone deacetylase HDACs and its inhibitor HDACi for the treatment of pulmonary fibrosis. Through this study we intent to observe the effect of not selective histone deacetylase inhibitor TSA on bleomycin induced pulmonary fibrosis in rats in vivo experiments and further explore the possible mechanism.

Material and methods

Materials

46 healthy male SD rats weight 160-180g with SPF conditions were purchased from Shanghai silaike experiment animal limited liability company and raised in a clean environment laboratory animal center of Shanghai University of Traditional Chinese Medicine. Trichostatin A was purchased from Sigma Company. Bleomycin was purchased from Nipponkayaku.Co Japan.Antibodies to p-SMAD2 and $HDAC_2$ were purchased from Santan Cruz Company. The second antibody was goat-anti-rabbit IgG purchased from Genscript Company. Primers for $HDAC_2$,and SMAD2 were synthesized by Shanghai Branch of Invitrogen Company. Image pro-plus for windows was purchased from Media cybernetics, USA.

Methods

Experimental animals and treatment: 46 SPF level male SD rats were randomly divided into four groups: ten for normal control group, fourteen for model control group I, twelve for model control group II and ten for treatment group. Rat pulmonary fibrosis was induced by bleomycin(5mg/kg) via single intratracheal perfusion in the two model control groups and treatment group. Normal control group was instilled with a corresponding volume of 0.9% saline intratracheally. Treatment group was treated by the dilution of TSA 2mg/kg DMSO 60ul and0.9% saline 1.2ml intraperitoneal injection from the next day ,once a day for three days. Model control group II was treated by the dilution of DMSO 60ul and0.9% saline 1.2ml intraperitoneal injection from the next day once a day for three days. Model control group I and normal control group were treated by 0.9% saline 1.2ml intraperitoneal injection from the next day once a day for three days. The death were no for normal control group ,five for model control group I ,four for model control group II and two for treatment group. All the animals were sacrificed on the 21 day after modeling for the lung tissue and serum.

Under 10% chloral hydrate intraperitoneal anesthesia, rats were opened the abdominal cavity. Turned intestinal tract to the other side, and wipe

the abdominal aorta with a cotton ball gently . Holding the 10ml syringe needle into the abdominal aortic bifurcation, pump slowly 7-8ml into blood coagulation promoting tubes under the condition of 4 , and centrifugal at the speed of 3500 rpm 15min,and take the supernatant 1-2ml into EP preserved in -20 oC refrigerator for further Western blot measurements. Rapid thoracotomy dissection from lung, middle lobe of right lung was fixed in 10% neutral formalin for 24 hours, embedded in paraffin, 5um sections perform HE and Masson staining, the remaining lung tissue was then used for mRNA measurements.

Real-time PCR on lung tissue of rats: Total RNA was extracted according to methods provided by Invitrogen Company Trizol kit. cDNA were synthesized with reverse transcription reaction, which were as template to be amplified and fluorescence quantitative detection was performed using FTC2000 fluorescent quantitative PCR system. The ratio of relative copy number of the target gene divided by the reference gene relative copy number was as the quantitative data.

Sequences of primer pairs were as follows: rat $HDAC_2$ sense, 5'-TGCGCTTGCCCTGGCCTCAG -3',and rat $HDAC_2$ antisense,5'-AGGAAGGGCCCTGGTGTAGTAGGAG -3' rat SMAD2 sense, 5'- TGCCGCCTCTGGATGACTA -3' and rat SMAD2 antisense, 5'-CGGAGAGCCTGTGTCCATACT -3'; rat GAPDH sense, 5'-ACAGCAACAGGGTGGTGGAC-3', rat GAPDH antisense, 5'-TTTGAGGGTGCAGCGAACTT-3'.

Western blot.

Tissue samples were prepared in a lysis buffer. After sonication and centrifugation, supernatants were collected, and the protein concentration was determined with the BCA protein assay kit (Sangon, Shanghai).Sixteen microliters of protein from each sample were separated by SDS-PAGE and transferred to nitrocellulose filter (NC) membrane (Whatman). After treatment with PBST buffer containing 5% skim milk at 4°C overnight, membranes were incubated with the following antibodies:$HDAC_2$ (Santan Cruz),p-SMAD2 (Santan Cruz). Blots were rinsed four times with PBST(phosphate-buffered saline, pH 7.3,with 0.05% Tween 20) and incubated with peroxidase-conjugated goat anti-rabbit (Genscript) for 2 h. Bound antibodies were visualized following chemiluminescence detection on autoradiographic film. The results are representative of at least two independent experiments.

Statistical analysis

All data are expressed as mean ± standard deviation (±s), using single factor analysis of variance. LSD and SNK test were used for Pairwise comparisons of each group with Homogeneity of variance, while Dunnett' s T3 test with heterogeneity of variance. P <0.05 was considered statistically significant difference. The statistical software was SPSS16.0.

Results

Morphological observation of the lung tissue of rates: HE staining showed that in the normal control group, the alveolar structure was integral and the stromal showed no significant inflammatory infiltration or fibrous hyperplasia (Fig. 1) In the two model control groups, the alveolar septa became wider, a large number of alveolar collapse or even disappeared, and located bullae appeared. There was inflammatory infiltration in pulmonary interstitial (P<0.05 Fig. 2,3). There were no significant differences between the two model control groups (P>0.05). Treatment group had less alveolar septal

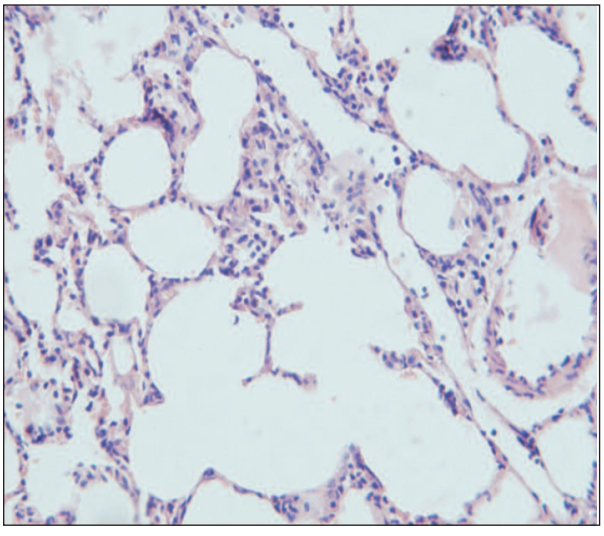

Fig. 1. Normal Control Group HE X 200

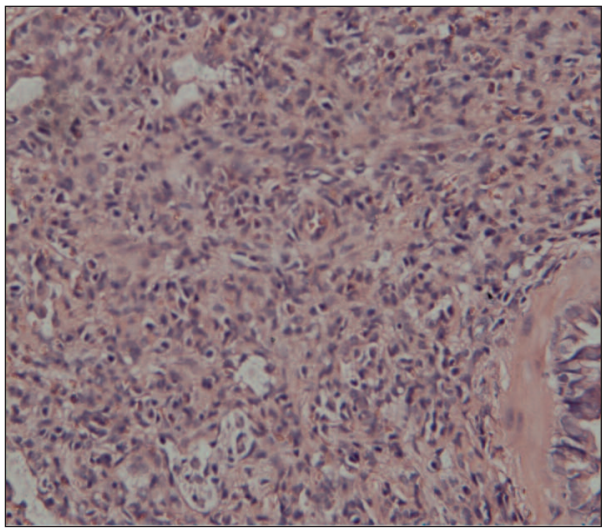

Fig. 2. Model Control Group I HE X 200

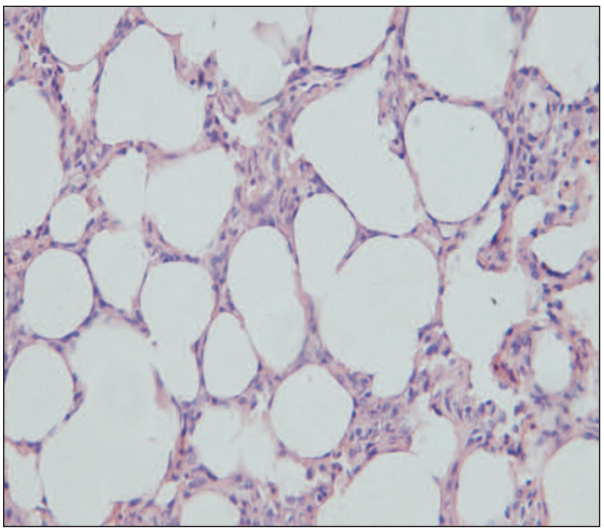

Fig. 4. Treatment Group HE X 200

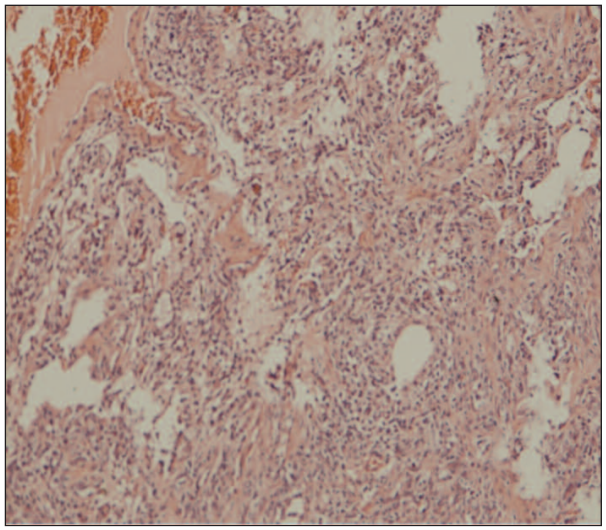

Fig. 3. Model Control Group II HE X 200

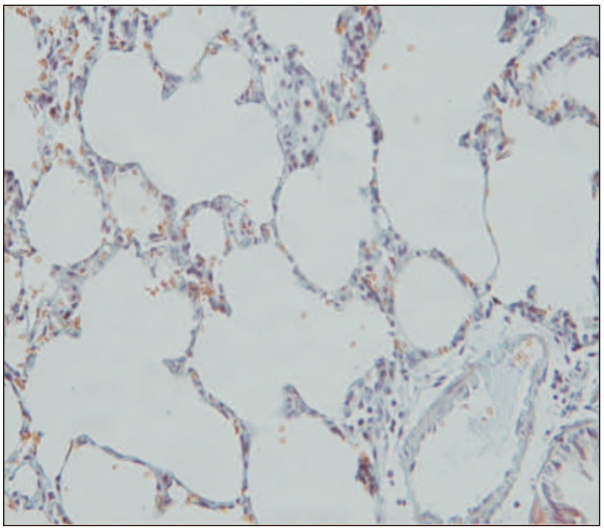

Fig. 5. Normal Control Group Masson X 200

thickening, interstitial inflammatory cells and fibrous hyperplasia compared to the two model groups (P<0.05 Fig. 4). But it had no significant difference compared with the control group (P>0.05)

Masson staining shows in the normal control group, the structure of lung tissue was normal and only under alveolar epithelial cells lined the normal green collagen fiber scaffolds with no inflammation response(Fig. 5). In the two model groups, a lot of green collagen was deposited in interstitial lung, bronchial and perivascular with structure of lung tissue disorder and alveolar large collapse (P<0.05 Fig. 6,7). There were no significant differences between the two model control groups (P>0.05). The treatment group had alveolar structure basically normal, less alveolar septal thickening and collagen fibers compared with the two model groups (P<0.05 Fig. 8). But the fibrosis degree of treatment group had no significant difference compared with the normal control group (P>0.05).

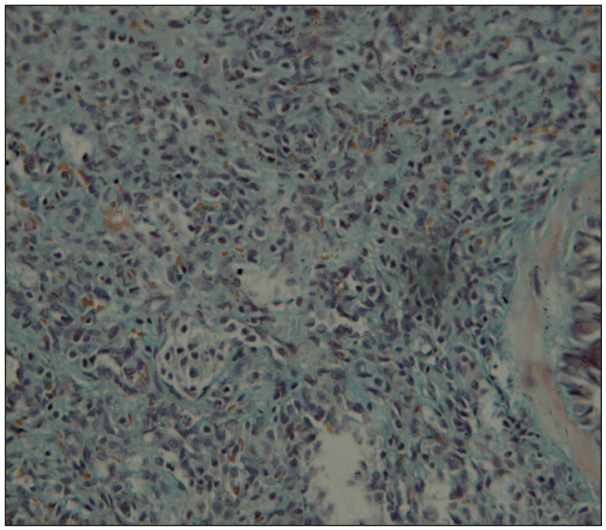

Fig. 6. Model Control Group I Masson X 200

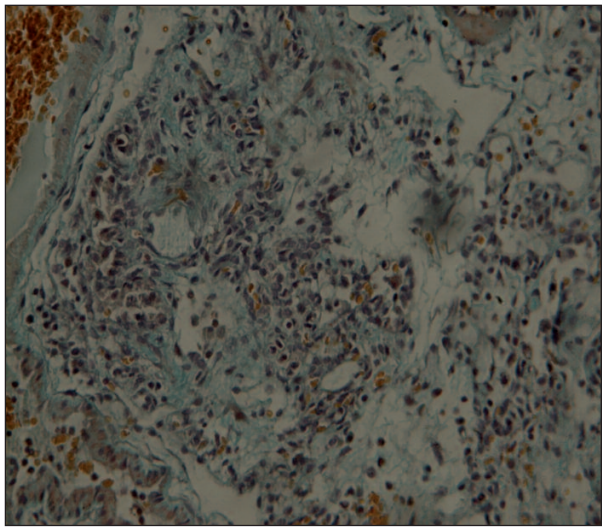

Fig. 7. Model Control Group II Masson X 200

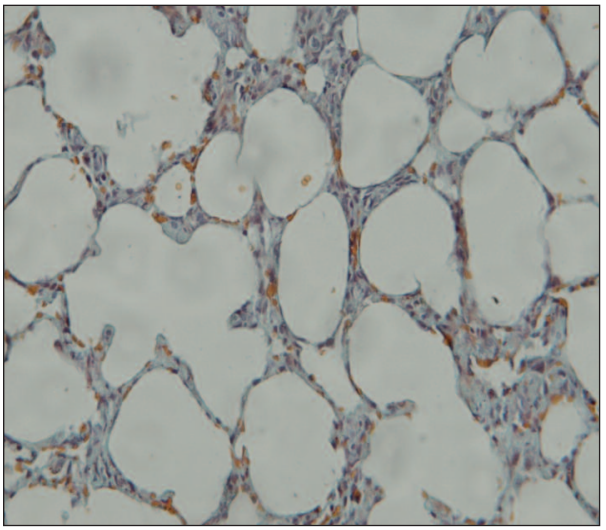

Fig. 8. Treatment Group Masson X 200

$HDAC_2$ mRNA, SMAD2mRNA expression in lung tissue of rats(Table 1): The $HDAC_2$mRNA expression was decreased in treatment group compared with other three groups (P<0.05), but increased in two model control groups (P<0.05). The SMAD2mRNA expression was increased in treatment group and two model control groups compared with normal control group (P<0.05). But there were no significant differences in the SMAD2 mRNA expression between treatment group and the two model control groups (P>0.05).There were no significant differences in the $HDAC_2$mRNA, SMAD2mRNA expression between the two model control groups (P>0.05).

The serum protein content of $HDAC_2$ and p-SMAD2 of rats in Western blot: The protein content of $HDAC_2$ was decreased significantly in treatment group than in the two model control groups and nor-

Table 1. $HDAC_2$ mRNA, $SMAD_2$mRNA expression in lungtissue of rats in each groups (X±s)

groups / content	Normal control group	Model control group I	Model control group II	treatment group
$HDAC_2$	0.5594±0.1613	2.9773±0.9018 ▼	3.4777±0.9330 ★	0.2890±0.0357 ◆
SMAD2	0.1943±0.0338	0.3696±0.0747 ▼	0.4043±0.1226 ★	0.537±0.2021 ●

Note: n=4. ▼ indicates comparison of $HDAC_2$mRNA and $SMAD_2$ mRNA between two model control groups and normal control groups. P<0.05. ★ indicates that there was no significant difference between two model control groups (P>0.05). ◆indicates that $HDAC_2$mRNA was decreased in treatment group compared to normal control group P<0.05. ● indicates there was no significant difference in SMAD2mRNA between treatment group and two model control groups P>0.05.

mal control group($p<0.05$; Fig. 9),and it was increased in the two model control groups compared to normal control group($p<0.05$; Fig.9). The protein content of p-SMAD2 had no significant differences between treatment group and two model control groups ($p>0.05$; Fig.9),which was lower in normal control group($p<0.05$; Fig. 9).

Discussion

Pulmonary fibrosis is a series of diffuse pulmonary diseases characterized by chronic diffuse interstitial pulmonary fibrosis and pulmonary physiological dysfunction, which is mainly manifested as diffuse alveolar inflammation, lung injury, lung excessive tissue repair and fibrosis. Lesions from inflammatory cells (mainly monocytes, macrophages) infiltration, alveolar epithelial cells, mast cells, endothelial cells and interstitial cells (such as fibroblasts, myofibroblasts) and it gradually cause alveolar damage of epithelial cells and endothelial cells, then induce the deposition of extracellular matrix protein ECM and collagen and finally resulting in pulmonary tissue damage and loss of function (6).

In the rat model of pulmonary fibrosis, many fibrogenic cytokines and growth factors leading to imbalance of synthesis and degradation of collagen, as

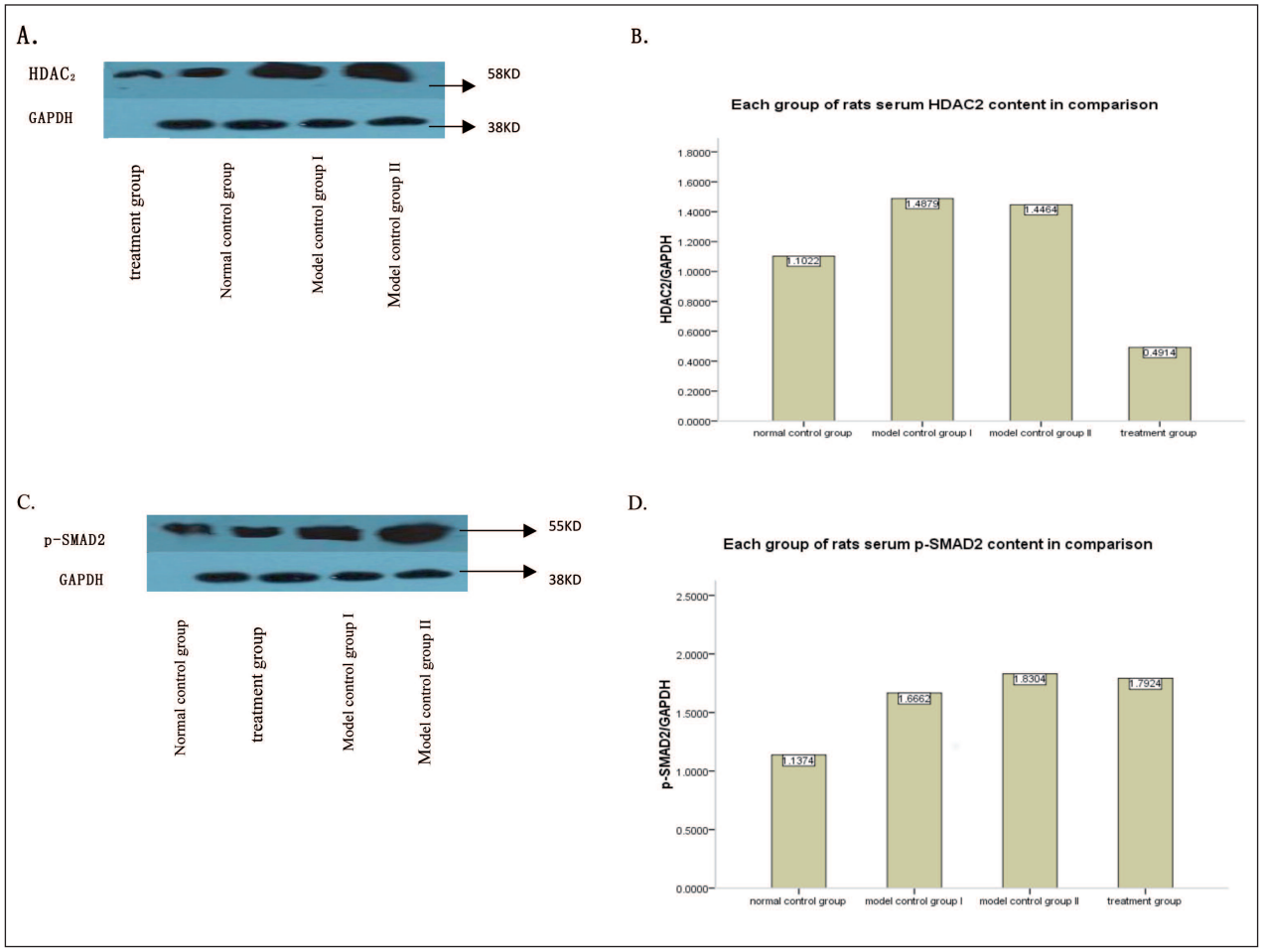

Fig. 9. Each group of rats serum $HDAC_2$ and p-SMAD2 content in comparison with Western Blot..
Treatment group(TSA)had less $HDAC_2$ (A,B)content comared to other three groups($p<0.05$).But it was increased with serum p-SMAD2 content comared to normal control group($p<0.05$)(C,D).And it had no significant differences between two model control groups. ($p>0.05$)(A,B,C,D). Two model control groups both had higher serum $HDAC_2$ and p-SMAD2 content than normal control group ($p<0.05$)(A,B.C,D).

well as fiber cell number increasing and collagen synthesising, result in excessive collagen reconstruction of pulmonary interstitial. The lung tissue pathological grade reflects mainly the level of pneumonia cell infiltration and fibrosis. The experimental results showed that the inflammatory cell infiltration and fibrosis degree of lung tissue in TSA treatment group were reduced compared with two model control groups. So TSA can inhibit pulmonary fibrosis process of rats induced by bleomycin.

Histone acetyltransferase (HATs) and histone deacetylases (HDACs) are considered as important enzymes which included in mechanism in the regulation of gene transcription. If the balance of HAT and HDAC is destroyed under certain conditions, the imbalance of gene transcription and abnormal gene expression will occur. Recent studies suggest, HAT / HDACs imbalance has related to disorders of tissue fibrosis (7-10). According to the analysis of different HDACs structures homologous of yeast phylogenetic (11), $HDAC_2$ belongs to class I HDACs which had homology with yeast Rpd3 gene, located in the nucleus. It also includes $HDAC_1$, $HDAC_3$, $HDAC_8$. The catalytic site in the class I HDACs contains zinc ions, which can be inhibited by HDAC inhibitor trichostatin A (TSA). Also in treatment group we got the result that it had lower serum protein of $HDAC_2$ and $HDAC_2$mRNA expression in lung tissue than the two model control groups. So it indicated that TSA attenuates pulmonary fibrosis which may be related to the inhibition of $HDAC_2$ expression at the protein level.

As we know, in the rat model of bleomycin induced pulmonary fibrosis,TGF-β is a multifunctional cytokine which plays an important role in in regulating cell growth, differentiation, apoptosis and interstitial synthesis. TGF-β induces signal from the cell membrane into the nucleus, and have related to the regulation of the gene expression of Smad family proteins (12,13). The Smad family proteins are intracellular kinase substrate of cytokine receptors which induce the regulation of gene expression of TGF-β (14). Smad2 is receptor activation protein in combination with the type I receptor of TGF-β to form receptor complex. Released from the receptor complex through phosphorylation, it is combined with the cytoplasm Smad4 to form oligomeric complexes. And then the complexes transfer to the nucleus with activating promoter of extracellular matrix components such as various type of collagen, thereby increasing the synthesis of extracellular matrix. Study of Duan Huijun (15) in animal models of renal interstitial fibrosis had confirmed this mechanism of TGF- 1/ Smads signal transduction, and also confirmed that by phosphorylation Smad2 are activated to p-Smad2 and then the deposition of extracellular matrix was promoted. Study (16,17) showed that TGF- 1 in vivo and in vitro can both stimulate fibroblasts differentiate into myofibroblasts (via SMAD2 phosphorylation pathway).Above experiment results mainly indicates that through down-regulation of HDAC expression TSA had its anti-fibrosis effect. In order to explore the relationship between TSA and SMAD2, the expression of SMAD2mRNA in lung tissue and the serum protein concentration of p-SMAD2 in rats were determined in this experiment. After modeling, SMAD2mRNA expression in lung tissue and serum protein concentration of p-SMAD2 in rats were increased compared to normal control group, which were consistent with rat lung pathology. But the index in TSA treatment group had no significant difference compared with two model control groups which indicated that TSA with inhibition of pulmonary fibrosis induced by bleomycin have no related to phosphorylation pathway of SMAD2.

The results suggest that TSA can reduce pulmonary fibrosis induced by bleomycin in rats. But as we know, fibrosis in body is a process of multiple factors and multiple factors participation, and mechanism of histone deacetylase inhibitors inhibited the pulmonary fibrosis remains to be further deep study.

Conflict of interest statement:

We declare that we have no conflict of interest.

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