

WITHDRAWN: Antitumor Effect of Membrane-type PBLs on Non-Small Cell Lung Cancer Cell of ICR Mice

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Research

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EDITORIAL NOTE:

The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

This study aims at probing the inhibitory effect of transmembrane PBLs on non-small cell lung cancer (NSCLC) H446 cells and the potential application of PBLs on immune system of the experimental mice loaded with H446 cells. The changes of the gene expression of microRNA-25 and 223 in ICR mice with NSCLC were also investigated. Sixty ICR mice were randomly divided into experimental and control groups. The animal model was established via inoculation of NSCLC H446 cells at the hind thigh of mice. The plasmid PBLs was dissolved in saline solution and injected into the muscle of left thigh of the mice in experimental groups with different doses (0.1 mg, 0.2 mg and 0.3 mg per ICR mouse) using in situ injection method. After injection of PBLs solution, each three mice were killed at 12 h, 24 h, 36 h, and 48 h, respectively. The expression of microRNA-25 and 223 were detected by semi-quantitative reverse transcription-polymerase chain reaction. Tumor Necrosis Factor- γ (TNF- γ), Interleukin-2 (IL-2) and Heat Shock Protein 70 (HSP70) in Bronchoalveolar Lavage Fluid (BALF) were detected by enzyme-linked immunosorbent assay. The expression of TNF- γ and IL-2 protein in lung tissue were detected by western blotting. The expression of microRNA-25 was up-regulated in the tissues and BALF with a dose- and time-dependent manner while microRNA-223 was down-regulated. The difference were statistically significant comparing the control group ($P<0.05$). The TNF- γ and IL-2 levels in BALF of ICR mice in experimental group were increased comparing the control group with a dose-dependent manner ($P<0.05$). Synergistic effect between PBLs and HSP70 was also studied. It was found that the growth of tumor was significantly suppressed after the transfection of PBLs. In the presence of PBLs, the proliferation of splenocytes and cytotoxicity in early phase of tumor development was significantly enhanced. Thus, such anti-tumor effect was further improved by the synergistic effect of PBLs with HSP70. The expression of microRNA-25 and 223 are associated with NSCLC in a dose- and time-dependent manner, they might be considered as potential biomarkers for early diagnosis of NSCLC.

Introduction

Lung cancer, as one of the common malignancies, can be divided into two categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [1, 2]. The latter is the most general pathological type of lung cancer. Basically, lung cancer is a common malignant tumor that inflicts serious damage. In China, the morbidity rates of this disease are quite high. Lung cancer cells have been reported to have very strong invasion and migration abilities. The patients easily suffer relapse, as well as lung cancer cell migration and infiltration, which are life-threatening. The five-year survival rate of lung-cancer patients is only 16.8%, and 50%-60% patients cannot be treated by surgery [3–6]. Moreover, most of the NSCLC patients are insensitive to radiotherapy and chemotherapy, it is urgent to develop anti-cancer drugs or methods with high efficiency and low toxic side effects based on sub-biological research.

MicroRNAs (miRNAs) are small, stable RNAs that critically modulate post-transcriptional gene regulation. The miRNAs can be ubiquitously or variably expressed in different tissues and cell types and they are known to have cell type specificity [6]. At present, miRNAs have been reported as potential biomarkers for various types of disease. It was reported that miRNAs are present in human plasma in a remarkably

stable form that is not prone to RNase degradation and established the measurement of tumor-derived miRNAs in serum or plasma [7]. These discoveries generated enormous enthusiasm for the potential use of miRNAs as biomarkers of neoplastic and non-neoplastic disease. Many studies have reported that plasma miR-25 may be a candidate biomarker for early detection and diagnosis of cancer [8–10]. The miR-223 promotes endotoxin-mediated inflammation in endothelial cells and it can be released from dendritic cells and are subsequently taken up by recipient dendritic cells [11]. In summary, miR-25 and 223 are involved in inflammation and considered as potential markers. However, the expression of miR-25 and 223 of NSCLC is still unknown. The aim of this study was to understand the changes of the gene expression of miR-155 and 127 in ICR mice with NSCLC.

PBLs were found to act as a ligand for reverse transmission of signals to affect tumor immunity correspondingly [12–21]. In order to investigate this new mechanism of PBLs molecules, NSCLC H446 cell strains of ICR mice were used to build a solid tumor model in thigh muscle to study the inhibitory effect of PBLs molecule on NSCLC solid tumor and the influence on immune system of tumor bearing ICR mice.

Materials And Methods

Animals

Male ICR mice (age: 4–6 weeks, weight: 19–23 g) were purchased from Animal Center of The Fourth Hospital of Hebei Medical University. Animal experiments were conducted in accordance with the “Care and Use of Laboratory Animals” from the National Institutes of Health Guide. The study protocols were approved by the Ethics Committee of The Fourth Hospital of Hebei Medical University.

Materials

The cell strain of NSCLC H446 was purchased from Jining Shiye (Shanghai, China). TRIzol was purchased from Sigma-Aldrich (Shanghai, China). The reverse transcriptase and RNasin were purchased from YEASEN Biotech Co. Ltd. (Shanghai, China). Primer pairs for the amplification of cDNA coding for miR-25 and miR-223 were supplied by Takara Biomedical Technology Co. (Beijing, China). Anti-TNF- γ , anti-IL-2 and anti- β -actin in 1:1,000 dilution were obtained from Beyotime Institute of Biotechnology (Beijing, China).

PBLs expression and detection

Eighty ICR mice were taken to conduct the measurements. The plasmid PBLs was dissolved in saline solution and injected into the muscle of left thigh of the mice using in situ injection method. The mice were randomly divided into PBLs 0.1 mg, 0.2 mg, 0.3 mg groups and the control group with 20 mice in each group. After injection of PBLs solution, each three mice were killed after 12 h, 24 h, 36 h, and 48 h, respectively. RT-PCR method was applied to detect the expression of PBLs in the muscle tissue of ICR mice.

Tumor cell inoculation

NSCLC H446 cells (1×10^6 /mL) were inoculated into the muscle of left thigh of mice (0.1 mL per mouse). The mice were randomly divided into normal saline group (the blank control group), pcDNA group (the plasmid control group) and PBLs group (the treatment group) with 20 mice in each group. Plasmid was injected on alternate day after the second day of inoculation with each injection of 0.1 mL (1 mg/mL), the injection site was the position of inoculated tumor cells. These inoculated mice were dissected after two weeks, the tumor was taken and weighed to observe the growth of tumor.

Based on above experimental groups, HSP70 group (the treatment group) and PBLs/HSP70 group (the combined treatment group, injection was half for each plasmid) were added with 20 mice in each group, NSCLC H446 cells were inoculated with the same method and the same treatment was conduct for examination of synergistic effect of PBLs with HSP70.

Sample collection

The ICR mice were killed at 12 h, 24 h, 36 h, and 48 h after anesthesia (2% isoflurane inhalation) employed. The fasting tissue and Bronchoalveolar Lavage Fluid (BALF) samples from the subjects were collected in containing vacationers, and transferred into a micro tube. The samples were centrifuged at 1,000 rpm and 4 °C for 10 min. Tissue and BALF sample were collected carefully and aliquoted in RNase-free tubes and stored at -80 °C for future use. The gene expression of miR-25 and 223 in lung tissue and BALF were determined by semiquantitative Reverse Transcription - Polymerase Chain Reaction (RT-PCR). Tumor Necrosis Factor- γ (TNF- γ), Interleukin-2 (IL-2) and Heat Shock Protein 70 (HSP70) levels of BALF were detected by Enzyme-linked immunosorbent assay (ELISA). The expression of TNF- γ , IL-2 and HSP70 protein in lung tissue were analysed by Western blotting.

cDNA synthesis

Single-stranded cDNA was prepared in a reverse-transcription reaction using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher, USA) using 5 μ g of RNA, according to the manufacturer's protocol. Reverse transcription primer,

miR-25: 5'-CTCAACTGGTGTGAGTCGCCCTC-3';

miR-223: 5'-ACACTCCAGCTGGAAAAGCTGGTT 3'.

Polymerase chain reaction

The PCR conditions were used as follows: initial denaturation at 95 °C for 5 min followed by 40 cycles, annealing at 95 °C for 15 s, and extension at 60 °C for 20 s and final extension at 72 °C for 40 s.

Gel electrophoresis

The PCR products of each interested gene were loaded into the same ethidium bromide-stained agarose gels (1%). A 1-kb DNA ladder molecular weight marker was run on each gel to confirm expected molecular

weight of the amplification product. Stained gels were recorded and the band intensity was evaluated using the Tanon 1600 software. Band intensity was expressed as relative absorbance units. The sample RNA was determined and calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization.

The ELISA assay

IL-2 and TNF- γ levels in BALF were measured by enzymelinked immunosorbent assay kit manufactured by Tiantan Biological Products co., Ltd (Beijing, China) according to manufacturer's instructions.

Western blot analysis

Lung tissues were harvested and homogenized using a homogenizer and tissue lysis/extraction reagent containing a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using a Bradford reagent (BioRad Laboratories, Inc.).

In vitro proliferation activity

Single cell was prepared under sterile conditions for determination of proliferation of the cells in vitro. The concentration of mice cell was adjusted to 1×10^6 /mL, suspended in culture medium containing 15% fetal calf serum followed by stained with CFSE. The solution was divided into two samples, one added NSCLC H446 antigen peptide and HSP70 protein complex. Both samples were transferred to a hole plate (100 μ L per hole) at 37 °C in an incubator for 7 days. Flow cytometry was used to detect the cell proliferation index.

Statistical analysis

Statistical analyses were performed using the SPSS software version 23.0, and all variables were expressed as mean \pm Standard Deviation (SD). Statistical significance between groups was analysed by the ANOVA when the variables between groups were normally distributed. A p value less than 0.05 was considered statistically significant.

Results

The expression of miR-25 in mice of induced lung cancer

To figure out the change of miR-25 expression of NSCLC H446 lung cancer in vivo, the membrane-type PBLs were administered into ICR mice through injection using established methods. Littermate neonatal rats were used as controls. At 12 h, 24 h, 36 h, and 48 h after injection, lung tissues and BALF were evaluated to determine the expression of miR-25. As shown in Figs. 1 and 2, for BALF the miR-25 levels were significantly increased after PBLs stimulation in PBLs groups. These results suggested that the expression of miR-25 increased with extension of time after injection of PBLs.

The expression of miR-223 in mice of induced lung cancer

As displayed in Fig. 3, as well as Fig. 4 into BALF-the miR-223 level of NSCLC H446 lung cancer were significantly decreased after PBLs stimulation in PBLs groups. Compared with the control group, miR-223 expression of lung tissue and BALF in the PBLs groups was significantly decreased with extension of time after injection of PBLs. There is statistical significance between the PBLs groups and the control group or among PBLs groups comparison ($P < 0.05$).

Pro-inflammatory cytokine production in mice of induced lung cancer

Inflammatory cytokines TNF- γ and IL-2 (Fig. 5) levels in the BALF increased with PBLs stimulation. Induction of PBLs resulted in a significant increase in TNF- γ production compared with the control group, moreover, IL-2 significantly increase by PBLs induction in a dose-dependent manner, the difference was statistically significant ($P < 0.05$). Consistently, inflammatory cytokines TNF- γ and IL-2 (Fig. 6) protein levels production in lung tissues were all increased in ICR mice. The more doses of PBLs, the higher TNF- γ and IL-2 level, the difference was statistically significant ($P < 0.05$).

Histological examinations of lung tissue

We also performed the Hematoxylin and eosin stain to examine lung pathological changes. As shown in Figs. 7–10, PBLs induced lung injury was promoted in experiment groups, as manifested by increase infiltration of neutrophils, increased thickening of interstitial alveolar regions, and minimal structural damage compared with that seen in the control group.

Effects of PBLs on enhancement of splenocytes proliferation and cytotoxicity

After inoculation for one week, the spleen cells were taken for proliferation experiment. Under the conditions of adding specific antigenic peptide stimulation and without specific antigenic peptide stimulation, flow cytometry was used to detect the mice splenocyte proliferation index of each experimental group. The results (see Fig. 11) showed that the PBLs group had significant difference ($P < 0.05$) comparing the saline control group and the pcDNA group with specific antigenic peptide stimulation. Also, the mice splenocyte proliferation index of the PBLs group without specific antigenic peptide stimulation did not have significant difference comparing the other two groups. The ratios of proliferation index obtained by adding specific antigenic peptide stimulation and without specific antigenic peptide stimulation for the three groups were 0.97, 0.99, and 1.62, respectively. This result indicates that the larger the ratio is, the higher the activity of spleen cell proliferation is under antigen stimulation.

The killing activity experiment of spleen cells further confirmed the specific killing activity of T cells. The specific killing function of PBLs group was stronger than pcDNA group ($P < 0.05$) and saline control group ($P < 0.01$). The killing rate was 32%, 23%, and 14%, respectively.

Two-color analysis of the expression of PBLs on mouse spleen B cells was shown in Fig. 11. ICR mice splenocytes were simultaneously stained with anti-mouse IgM[a] mAb DS-1 and purified anti-mouse PBLs

mAb 1D3. The staining of primary antibody was detected with FITC-conjugated goat anti-mouse Ig.

In vivo expression of HSP70 and PBLs in mice muscle tissues after 12 h, 24 h, 36 h, and 48 h were shown in Fig. 12. Western Blot used denaturing polyacrylamide gel electrophoresis (SDS-PAGE) for separation of protein mixture, the proteins were transferred to solid supports after separation and solid phase carrier absorb protein with non covalent interaction, which maintains the invariant properties of antigen protein or polypeptide onto membrane. Solid carrier protein or polypeptide behave as antigen and react with its corresponding antibody and enzyme labeled antibody to detect the expression of specific proteins through chromogenic substrate.

Synergistic effect of PBLs with HSP70

So far, we have demonstrated that transfection of PBLs into tumor-bearing mice significantly inhibited the growth of tumor. Furthermore, the growth was further inhibited when PBLs and HSP70 were simultaneously transfected. After inoculation for two weeks, of the average weight of tumor was only 0.42 g, significantly lower than that of PBLs transfected mice (0.84 g) or HSP70 transfected mice (1.57 g) and normal saline group (1.61 g). The experimental group treated with combined PBLs and HSP70 has significant difference ($P < 0.05$), suggesting that PBLs and HSP70 have stronger synergistic antitumor effect.

Discussion

Previous studies demonstrate that PBLs has been used as the receptor molecule on the surface of T3 cells in the studies of membrane-type PBLs [5–7]. Generally, HSP70 is considered as the ligand of PBLs [8, 9]. The signal produced by synergistic effect of HSP70 and membrane-type PBLs can prevent the activation of the cell and negatively regulate the cell immunity. However, if membrane-type PBLs inhibits receptor only, soluble PBLs should enhance the immune response by blocking effect. Previous studies have found that soluble PBLs does not produce an immune enhancing effect, but generates an immunosuppressive effect [10–12]. In addition, some studies have found that membrane-type PBLs is not only expressed on the surface of cells, but also on the surface of other tissue cells, including tumor cells [13, 14]. The function of expression of membrane-type PBLs in these cells is unclear. However, all these studies suggest that PBLs might have multiple biological effects.

In this study, it was found that T cells can enhance the immune function by transfecting the recombinant membrane-type PBLs eukaryotic expression plasmid into NSCLC H446 cells of vaccinated mice, which had significant antitumor effect. This result is not consistent with a previous publication which reported that membrane-type PBLs can induce T cell immune tolerance. The membrane-type PBLs is a receptor of the T cell surface, after binding with ligand for signal transduction, PBLs produced a series of changes within the cell. In current work, intramuscular injection of recombinant carrier of membrane-type PBLs was directly conducted, this carrier effectively expressed in muscle cells but was difficult to transfect T cells. The possibility that membrane-type PBLs as cell surface receptor expression in mature muscle cell surface to induce the produce of positive immune effect by muscle cells is very small. Another possibility

is that the membrane-type PBLs has ligand properties, which produced positive immune regulatory effect through the interaction with receptor of immune cells surface. Because only HSP70 molecules are known to bind with membrane-type PBLs, the existence of other receptor molecules remains unclear. In this research, the method of local transfection was taken to allow the expression of membrane-type PBLs only in specific position - the thigh muscle cells of ICR mice. The free cells that can contact membrane-type PBLs or even generate biological effects include macrophages, T cells, B cells and granulocytes [18, 19]. We speculate that in this case the membrane-type PBLs may not have a direct effect on T cells, but indirectly activate T cells via activation of macrophages or antigen cells. Because HSP70 that combined with PBLs can express in a variety of tissue cells including macrophages, the PBLs molecule will react with these cells. The RT-PCR results obtained with pure NSCLC H446 cell samples showed very weak expression of HSP70 only, the possibility that PBLs molecules reacted with the NSCLC H446 cells locally inoculated can be excluded.

Conclusions

In this study, we have found that the membrane-type PBLs and HSP70 could have synergistic effect. HSP70 as a molecule having clear physiological functions can interact with its receptor (expressed on the surface of activated T cells) to maintain the activity of T cells or further enhance the activity and turn it into memory T cells. The transformation is independent and not restricted by other molecules, suggesting that the mechanisms of membrane-type PBLs signal is activation of T cells eventually, and produce synergistic effect with HSP70 based on the activation. After 2 weeks of tumor cells inoculation, HSP70 molecule started to function. Although there are T cells activation at this moment, the effect of PBLs molecules is not further strengthened, suggesting that PBLs molecules do not influence the functions of T cells after activation.

Declarations

Ethics approval and consent to participate

All the experimental procedures mentioned in the present study were approved by the Science Research Department (in charge of animal welfare issue) of The Fourth Hospital of Hebei Medical University (Shijiazhuang, Hebei, China) and First Hospital of Qinhuangdao (Qinhuangdao, Hebei, China). Ethical approval for animal surgery was given by the animal welfare committee of both hospitals.

Consent for publication

All authors have consented the manuscript been published.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare no conflicts of interest.

Funding

N/A

Authors' contributions

Junli Cao and Peng Su contributed equally to this work.

Acknowledgements

N/A

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Figures

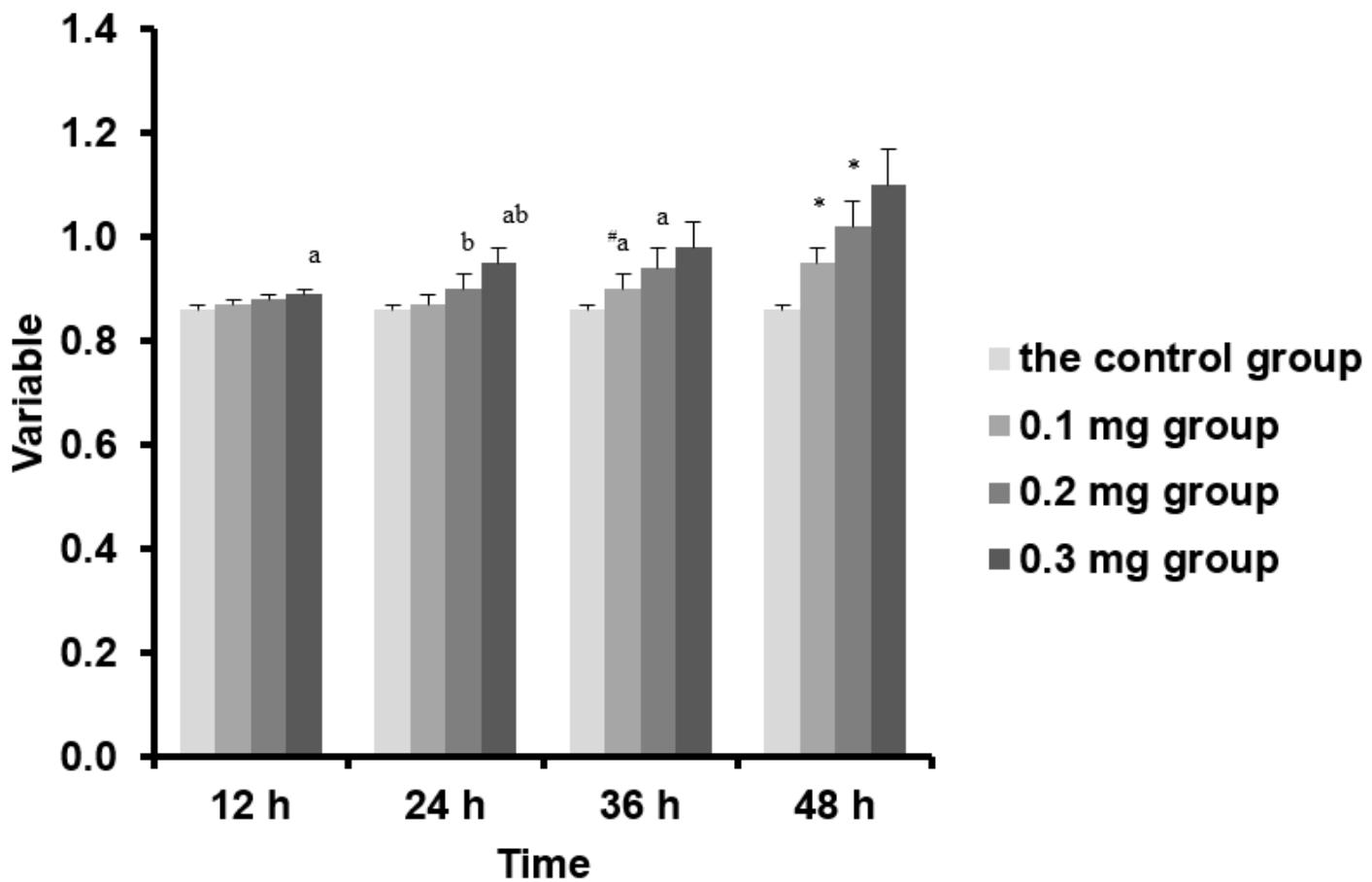


Figure 1

The expression of miR-25 of lung tissues. *P<0.05; significantly different from 12 h, #P<0.05; significantly different from 24 h, ΔP<0.05. Significantly different from the control group, aP<0.05; significantly different from the 0.1 mg group, bP<0.05; significantly different from the 0.2 mg group, cP<0.05.

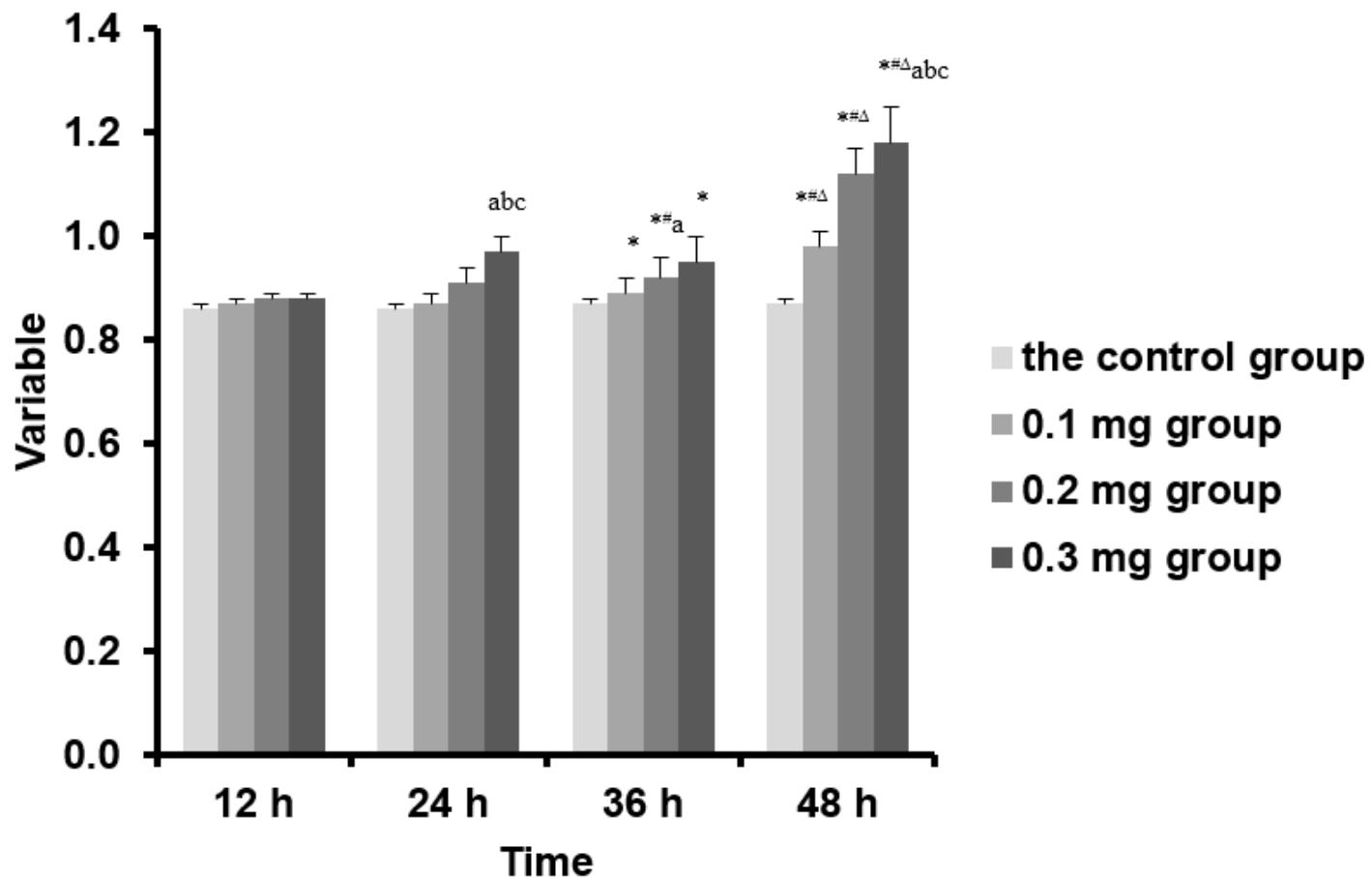


Figure 2

The expression of miR-25 in BALF. *P<0.05; significantly different from 12 h, #P<0.05; significantly different from 24 h, ΔP<0.05. Significantly different from the control group, aP<0.05; significantly different from the 0.1 mg group, bP<0.05; significantly different from the 0.2 mg group, cP<0.05.

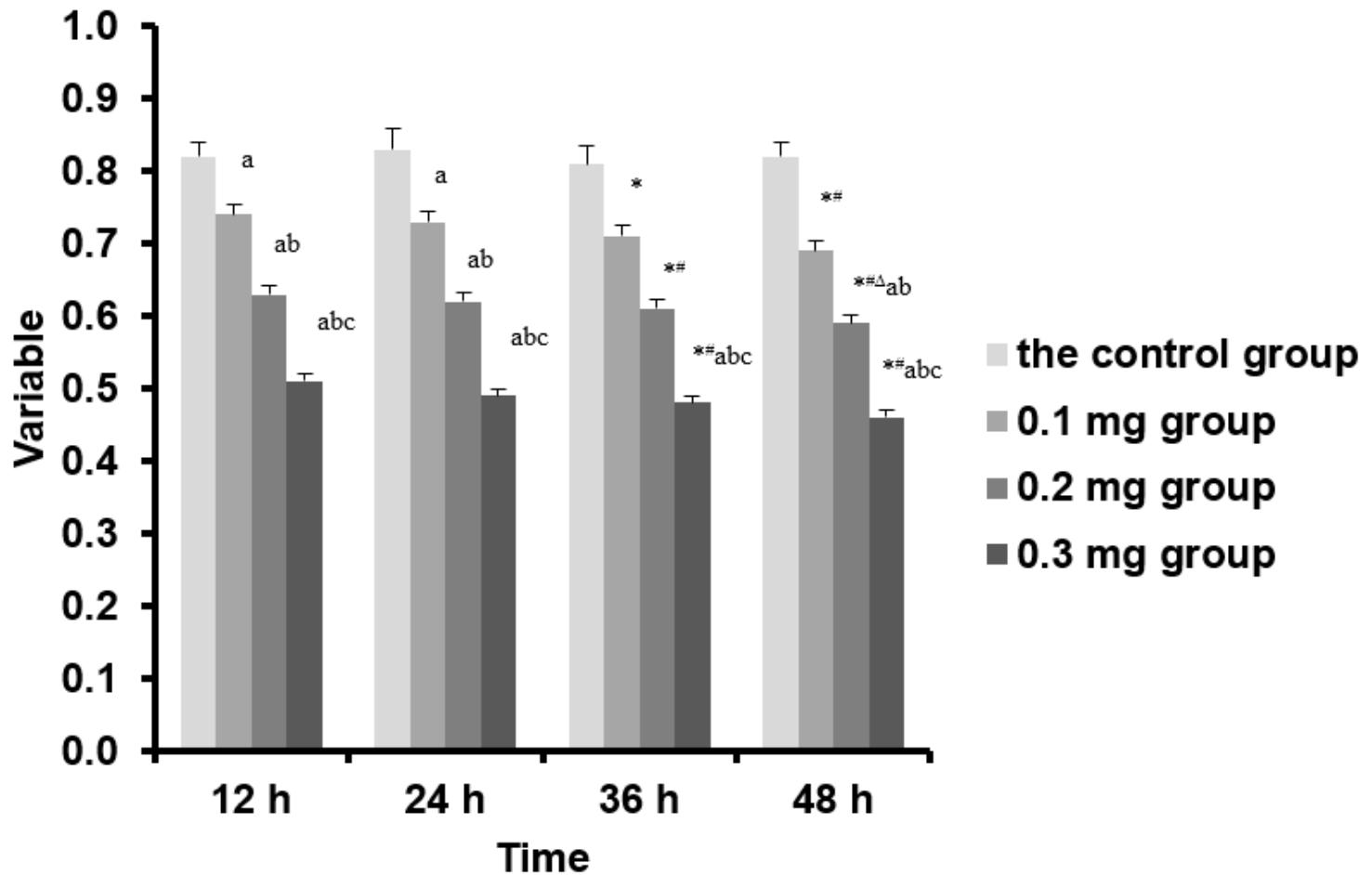


Figure 3

The expression of miR-223 of lung tissues. *P<0.05; significantly different from 12 h, #P<0.05; significantly different from 24 h, ΔP<0.05. Significantly different from the control group, aP<0.05; significantly different from the 0.1 mg group, bP<0.05; significantly different from the 0.2 mg group, cP<0.05.

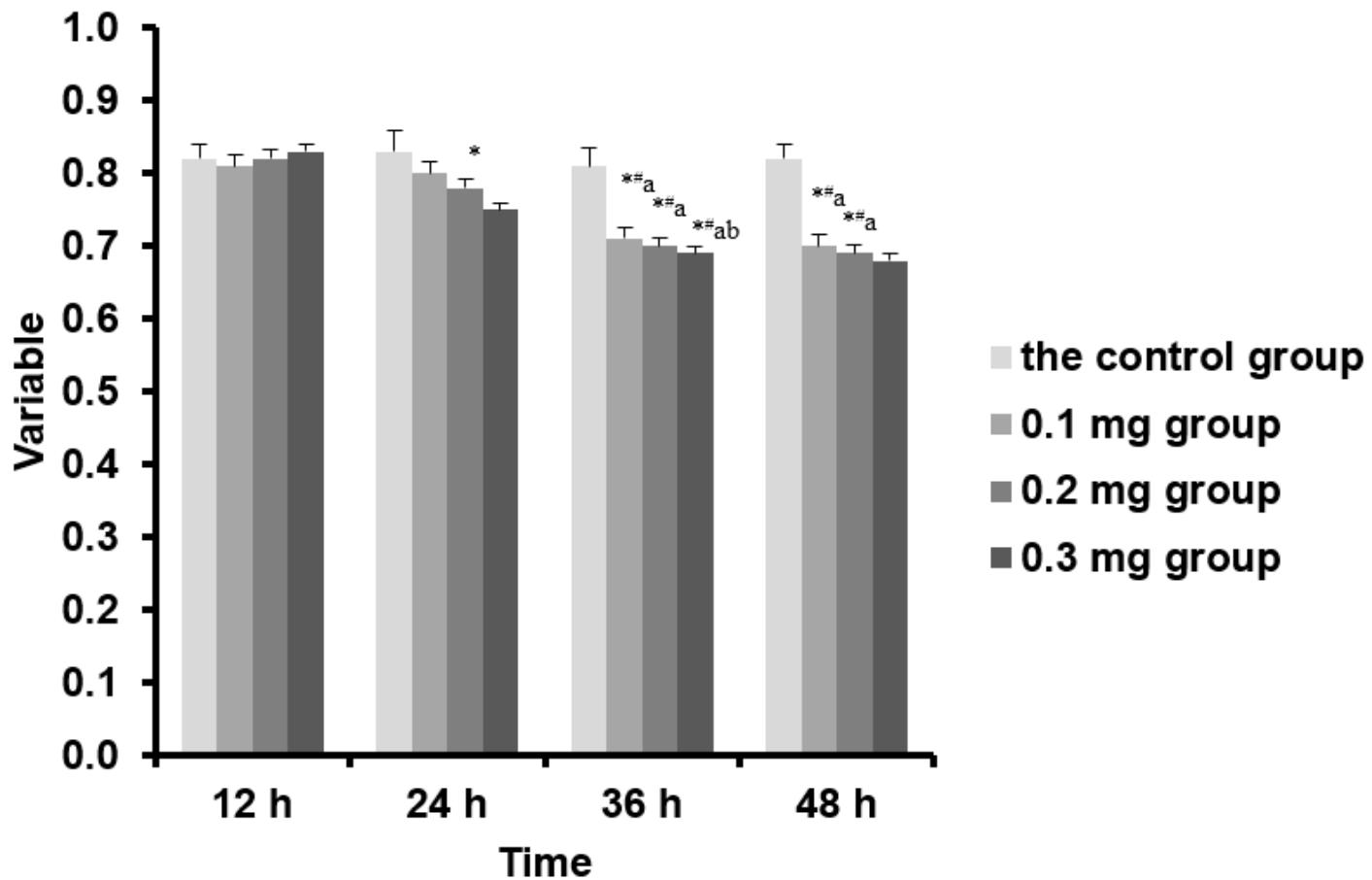


Figure 4

The expression of miR-223 in BALF. *P<0.05; significantly different from 12 h, #P<0.05; significantly different from 24 h, ΔP<0.05. Significantly different from the control group, aP<0.05; significantly different from the 0.1 mg group, bP<0.05; significantly different from the 0.2 mg group, cP<0.05.

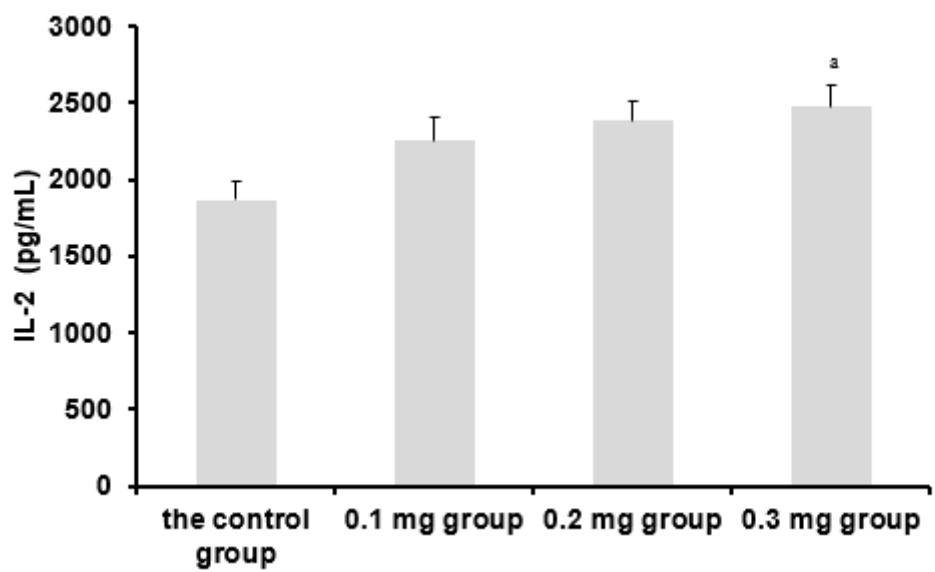
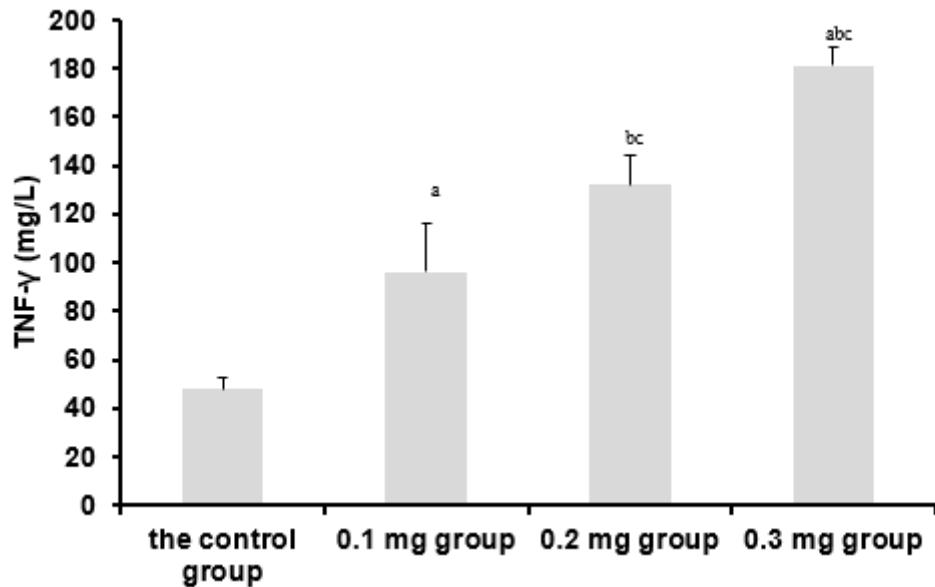


Figure 5

The expression of TNF- γ and IL-2 in BALF at 48 h.

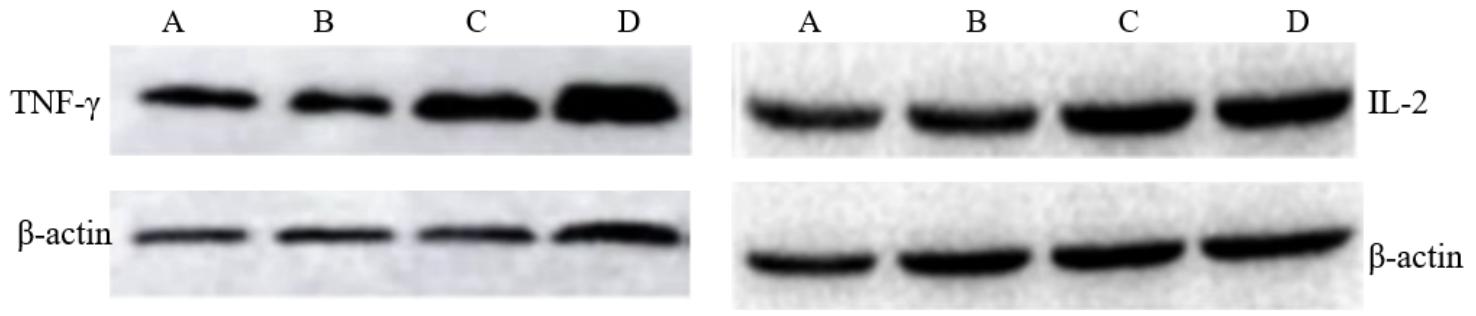


Figure 6

The expression of TNF- γ and IL-2 protein in each group. A: control group; B: 0.1 mg group; C: 0.2 mg group; D: 0.3 mg group.

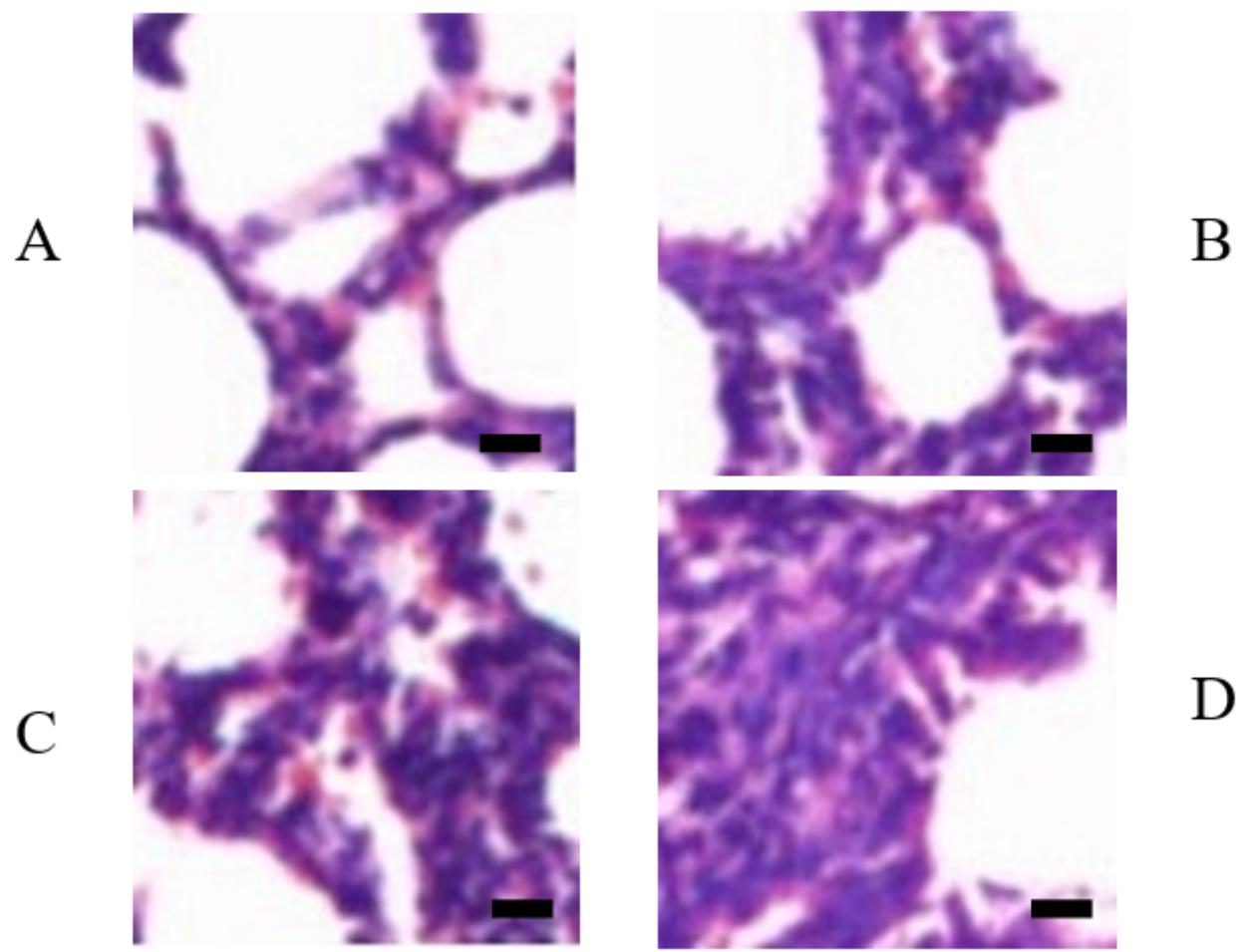


Figure 7

Histopathological changes of lung tissue in four groups at 12 h. A: control group; B: 0.1 mg group; C: 0.2 mg group; D: 0.3 mg group (scale bar = 10 μ m for all groups).

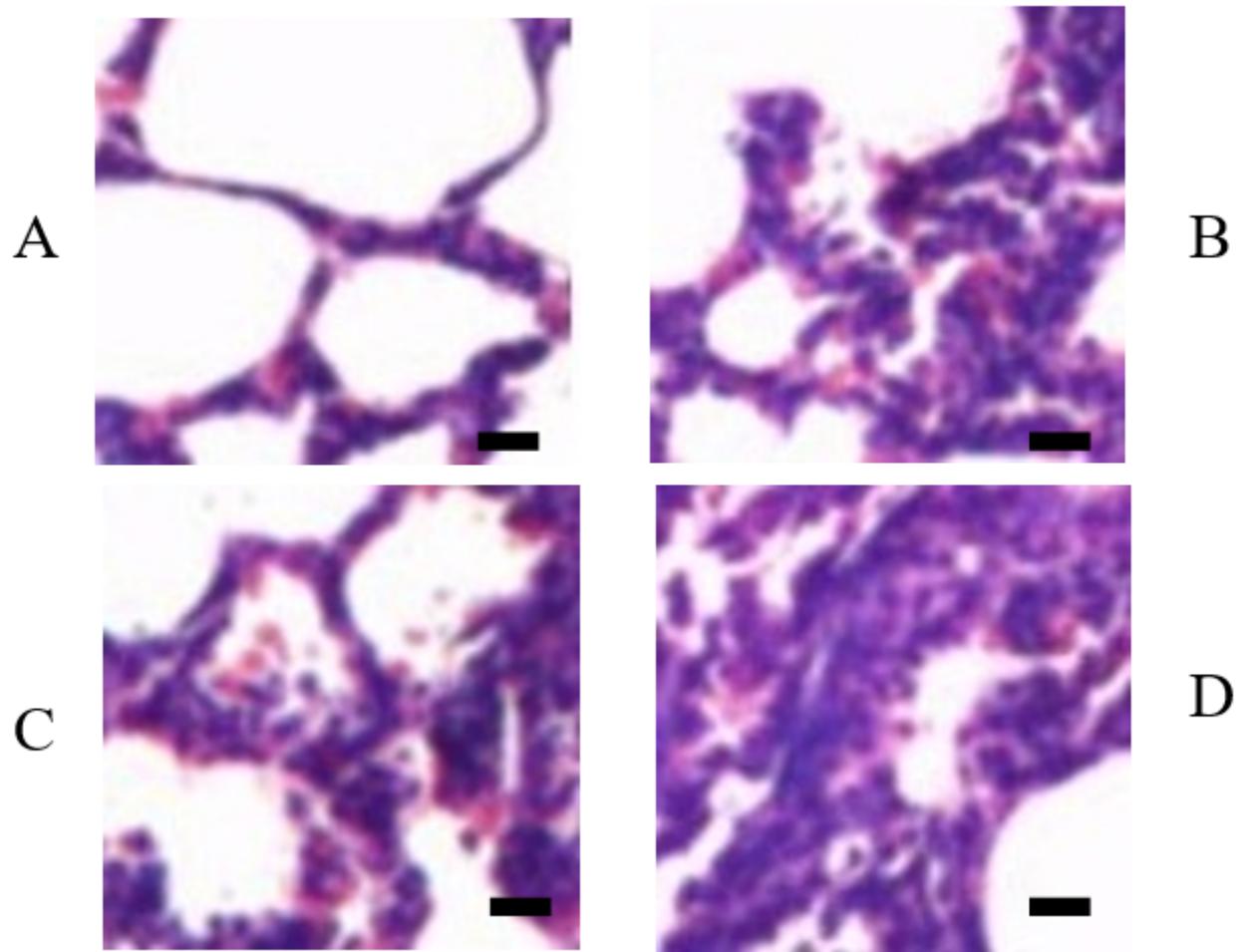


Figure 8

Histopathological changes of lung tissue in four groups at 24 h. A: control group; B: 0.1 mg group; C: 0.2 mg group; D: 0.3 mg group (scale bar = 10 μm for all groups).

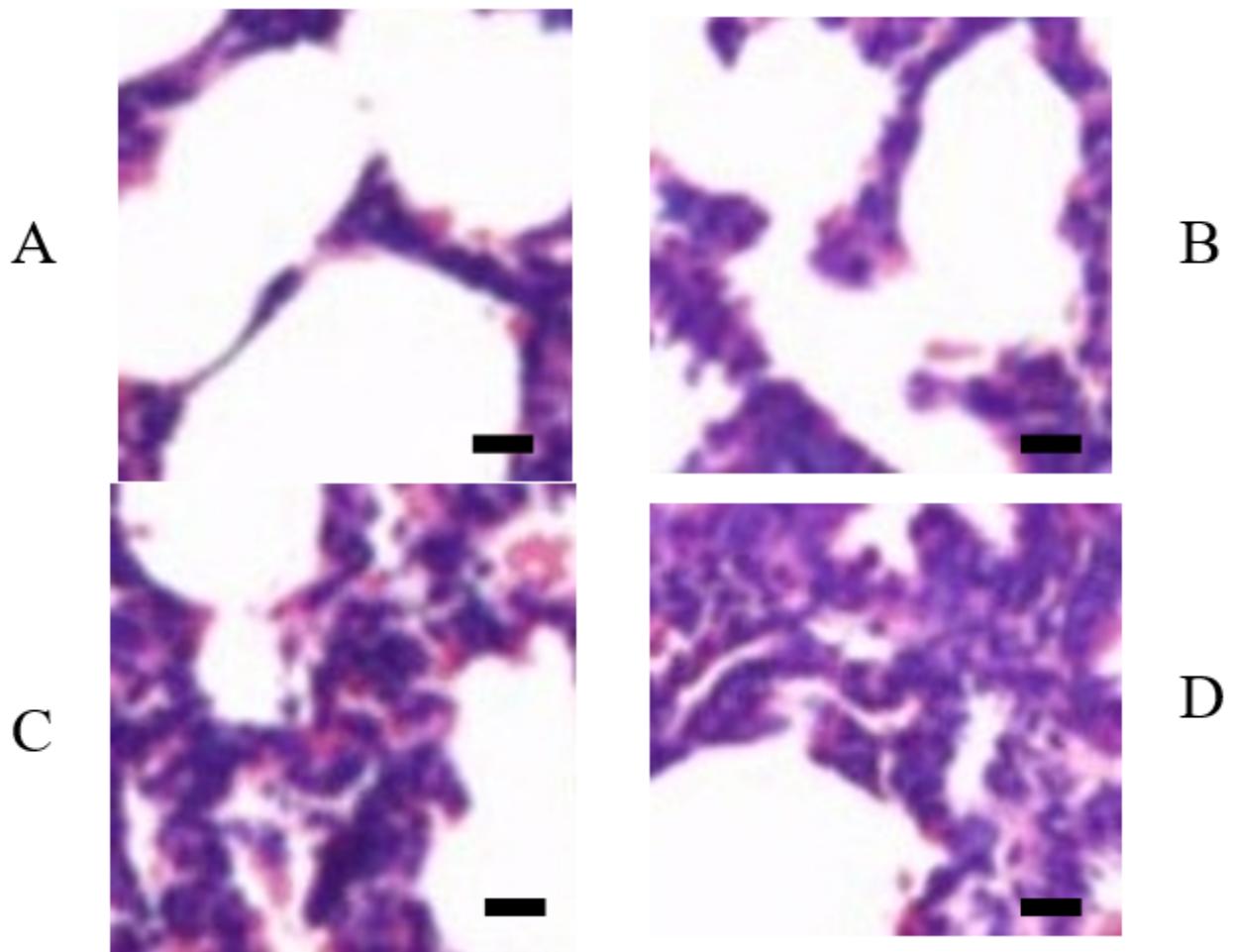


Figure 9

Histopathological changes of lung tissue in four groups at 36 h. A: control group; B: 0.1 mg group; C: 0.2 mg group; D: 0.3 mg group (scale bar = 10 μ m for all groups).

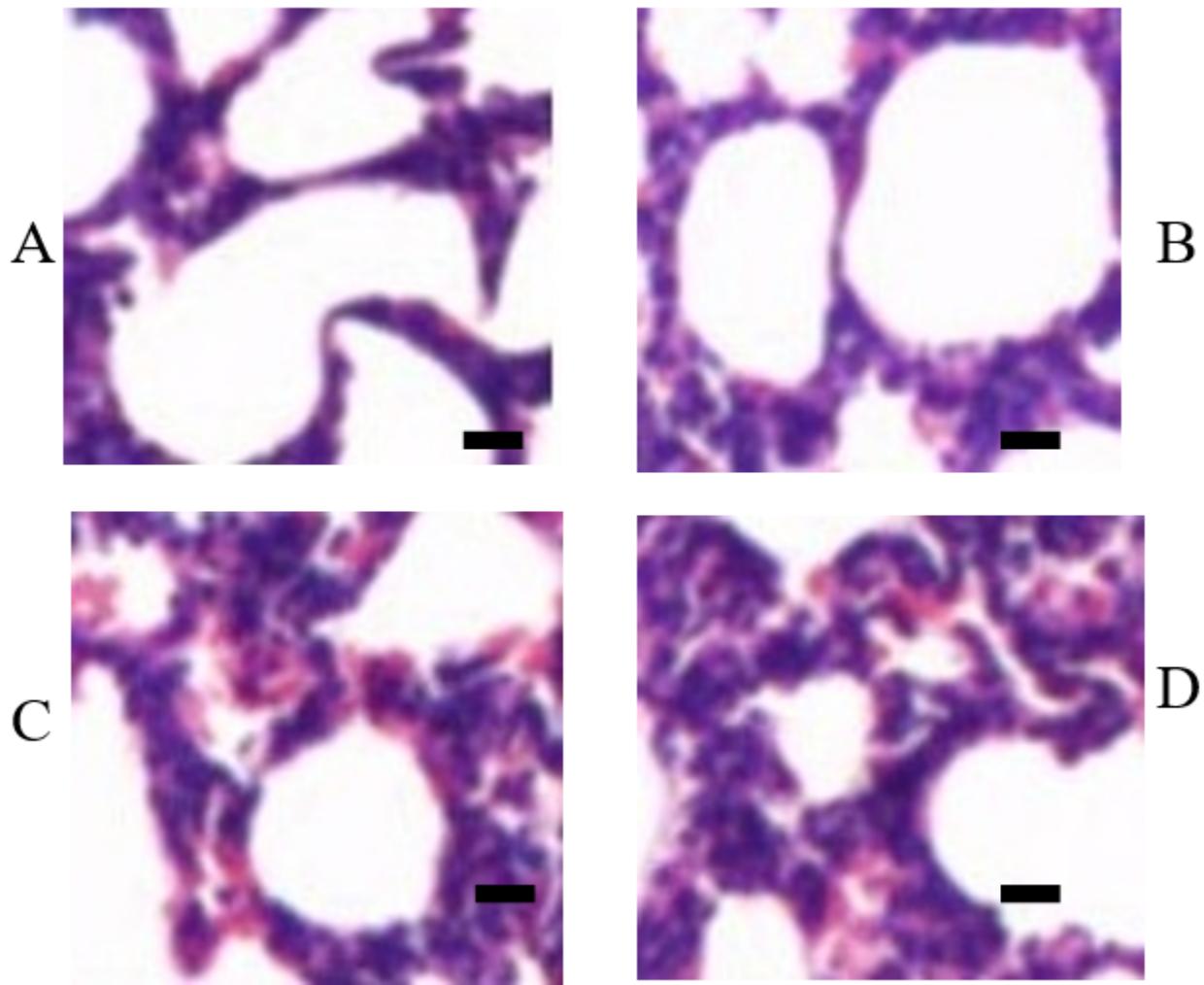


Figure 10

Histopathological changes of lung tissue in four groups at 48 h. A: control group; B: 0.1 mg group; C: 0.2 mg group; D: 0.3 mg group (scale bar = 10 μm for all groups).

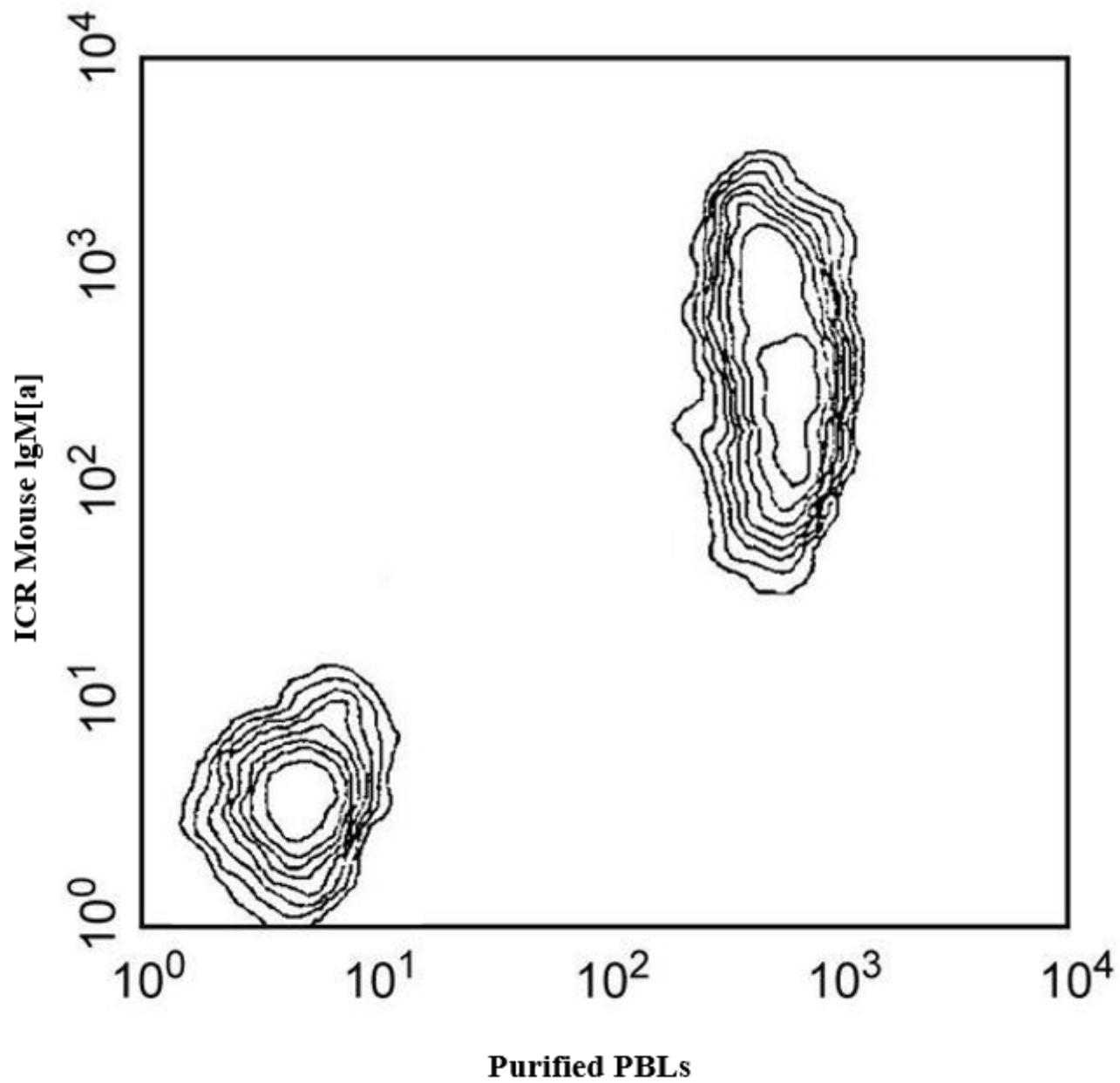


Figure 11

Flow Cytometry (FACS) image for PBLs.

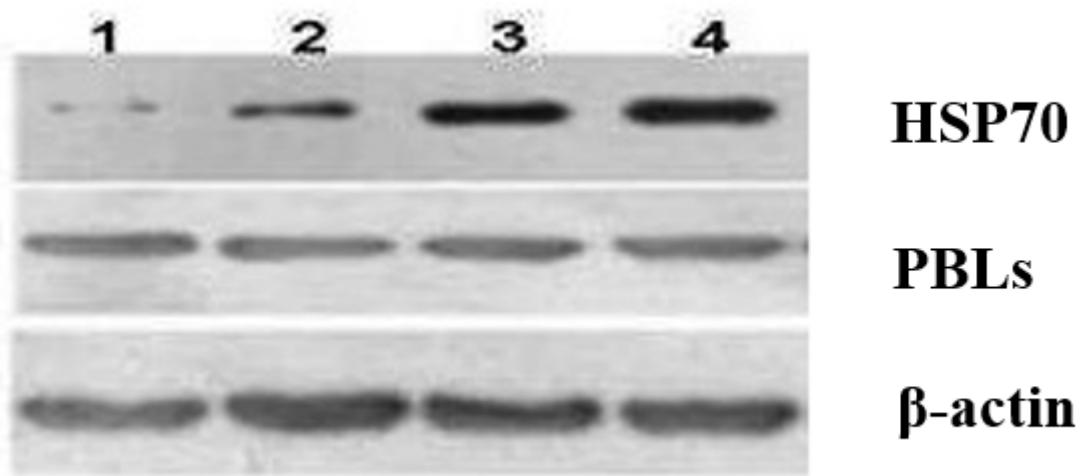


Figure 12

In vivo expression of HSP70 and PBLs in mice muscle tissues after 12 h, 24 h, 36 h, and 48 h.