Protective effects of pentoxifylline against chlorine-induced acute lung injury in rats

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Abstract

Objective

Chlorine is a chemical threat agent that can be harmful to humans. Inhalation of high levels of chlorine can lead to acute lung injury (ALI). Currently, there is no satisfactory treatment, therefore, effective antidote is urgently needed. Pentoxifylline (PTX), a methylxanthine derivative and nonspecific phosphodiesterase inhibitor, is widely used for the treatment of vascular disorders. The present study aimed to investigate the inhibitory effects of PTX on chlorine-induced ALI in rats.

Methods

Rats were exposed to chlorine. The histopathological examination were carried out. The effect of administration of PTX was evaluated. The expression of oxidative stress, hypoxia, and autophagy markers were examined by western blotting assay.

Results

The histopathological examination demonstrated that chlorine could destroy the lung structure with hemorrhage, alveolar collapse, and inflammatory infiltration. ROS accumulation was significantly higher in the lung of rats suffering from inhaling chlorine. The results also showed that PTX markedly alleviated the expression of lactate dehydrogenase (LDH), oxidative stress, and hypoxia. Additionally, PTX up-regulated the level of autophagy.

Conclusion

PTX could ameliorate chlorine-induced lung injury, thus suggesting that PTX could serve as a potential therapeutic approach for ALI.

Introduction

Chlorine, as a respiratory irritant, is widely used in numerous industrial processes, such as plastics, synthetic fibers, dyes, pesticides, and pharmaceutical manufacturing [1–2]. Injuries due to chlorine exposure are usually the result of accidents at swimming pools and the mixing of household agents [3]. Moreover, as a traditional chemical weapon, chlorine is still considered a terrorist threat [4–6]. In World War I, German troops released more than 150 tons of chlorine on April 22, 1915, in Ieper of Belgium. This attack killed up to 5,000 and caused injuries on both sides [7]. No matter accidental or deliberate, the release of chlorine poses a significant threat to public health [8–9]. Low concentration chlorine acts as an eye and oral mucous membrane irritant [10], but at the high level, it may induce damage to the lung, even resulting in acute lung injury(ALI) and acute respiratory distress syndrome(ARDS). Although there have

been a number of therapeutic interventions recognized over the past couple of years, there is still no specific antidote against chlorine poisoning [11]. Searching for novel drugs for the treatment of ALI induced by chlorine remains urgently.

Reactive oxygen (ROS) are known to contribute to the pathogenesis of ALI/ARDS, which may cause the endothelial and epithelial barrier dysfunctions [12–13].Through upregulating the expression of adhesion molecules, ROS may amplify the tissue damage and pulmonary edema. In a rats model of LPS-induced ALI, Duan et al. found that inhabited ROS might decrease the expression of adhesion molecules (ICAM-1 and VCAM-1), then attenuated ALI [14].

Hypoxemia is one of the main features of ALI. Hypoxia-inducible factor-1 (HIF-1) is an oxygen-dependent transcriptional activator that is widely expressed in tissue during hypoxia [15]. HIF-1α, the oxygen-regulated subunit of HIF-1, has been identified to play an important pathophysiological role in maintaining oxygen homeostasis. Under normal conditions, HIF-1α is degraded by ubiquitin-dependent proteasomal. When under hypoxia, the HIF-1α subunit is stabilized and accumulates in the nucleus, then regulates diverse processes [16]. Li et al. showed that emodin alleviated pulmonary inflammation in rats with LPS-induced ALI by inhibiting the mTOR/HIF-1α/VEGF signaling pathway [17]. HIF-1α signaling pathway may play a key role in the development of ALI.

Autophagy is a process of cell self-renewal that is dependent on the degradation of the cytoplasmic proteins or organelles of lysosomes [18]. Extensive work has been performed to confirm that autophagy is involved in the occurrence and development of ALI [19]. In the early stages (1 h and 2 h) of ALI induced by LPS, autophagy reached a peak at 2 h. As the ALI process progressed, autophagy decreased in a time-dependently manner [20]. However, the role of autophagy in ALI is still unclear. In cecal ligation and puncture (CLP)-induced septic mice, the emergence of autophagy alleviated the cytokine excessive release and lung injury, describing a protective role [21]. In vivo, autophagy aggravated oxidative stress in alveolar epithelial cells in H9N2 influenza virus infection [22]. However, there is no direct evidence for the effect of autophagy on chlorine-induced ALI.

Historically, the nonselective phosphodiesterase inhibitor pentoxifylline (PTX) is reported to be beneficial in vascular disease, including peripheral vascular disease, cerebrovascular disease, and a number of other conditions involving a defective regional microcirculation [23]. The beneficial effects are thought to be due to its anti-inflammatory properties by inhibiting the production of tumor necrosis factors [24]. More importantly, it is widely reported that PTX has been shown to inhibit liver ischemia/reperfusion injury, abdominal compartment syndrome, and intermittent hypobaric hypoxia in experimental animals due to its antioxidant function [25–26]. Recent research also implied therapeutic effects of PTX on a model of acid-induced ALI and endotoxin-induced ALI [27–30]. Furthermore, Mostafa-Hedeab et al. reported that PTX might exert a protective effects in COVID-19 [31]. However, as an effective drug candidate in the treatment of ALI induced by chlorine, it still needs to be explored.

Thus, this study aimed to investigate the potential effects of PTX on ALI induced by chlorine. Here, the status of oxidative stress, hypoxia, as well as autophagy in lung tissues were analyzed. The

characteristics of “ New use of old drugs” can be reflected on PTX.

Materials And Methods

Chemicals and reagents

Chlorine was obtained from Jinghua Gas Co., Ltd. (Changzhou, China). Pentoxifylline was provided by Sigma (St. Louis, MO). Kits for detecting the activity of lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), glutathione (GSH) and oxidized glutathione (GSSG) were supplied by Nanjing Jiancheng Bio-Engineering Institute Co., Ltd. Primary antibodies against VEGF, PTEN-induced putative kinase 1 (PINK1), Parkin and cytochrome-c oxidase subunit IV (COX IV) were brought from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against occludin and E-cadherin were bought from Abcam (Cambridge, MA, USA). Antibodies against SOD 2 and Beclin 1 were bought from CUSABIO BIOTECH CO., Ltd (Wuhan, China). Antibodies against HIF-1α, catalase (CAT), bcl-xl and β-actin were bought from Merck Millipore Technology (MA, USA), Proteintech Co., Ltd., (Wuhan, China), Cell Signaling Technology (Boston, USA) and Sigma (St. Louis, MO) respectively. Dihydroethidium (DHE) was purchased from Beyotime Co., Ltd. (Shanghai, China).

Animals and experimental design

A total of 24 adult male Sprague-Dawley rats (4-6 weeks old, weighing 200–220 g), were provided by the Experimental Animal Center of the Fourth Military Medical University. The animals were housed in cages (6 rats per cage) under a permanent temperature of 20–25 ℃ and a 12 h light/dark cycle. All the rats were allowed free access to food and water. Efforts were made to minimize animal suffering. According to a random number table, the rats were assigned to four experimental groups (6 rats/group). (1) normal control (NC) group; (2) chlorine group;(3) chlorine + PTX group; (4) PTX group. ALI was induced by inhaling chlorine (400 ppm) for 5 min. In addition, rats in the PTX and chlorine +PTX groups were intragastrically administrated with PTX (100 mg/kg) 30 min before chlorine exposure and treatment 15 min after chlorine exposure. The NC and chlorine-treated groups were orally administered with equal amounts of normal saline at the same time.

Histologic examination

The middle right lung lobes of the rats were fixed in 4% formaldehyde for 24 h. After dehydrated, the sections were embedded in paraffin and sliced at 3 μm. Following deparaffinized and dehydrated, the sectioned tissues were stained with hematoxylin (5 min) and eosin (1-2 min) (H&E). A light microscope (BX51; Olympus Corporation, Japan) was used to observe the extent of histological lung injury.

Detection of ROS formation

According to the previously described method [32], the intracellular ROS level was detected using the fluorescent dye DHE. Then, the tissue was collected and incubated for 30 min at 37 °C in the dark with 10

μM DHE and 10 μM Hoechst. After washed 3 times with PBS, the tissues were immediately observed by a laser scanning confocal microscopy (FV10i; Olympus Corporation, Japan).

Preparation of bronchoalveolar lavage fluid (BALF)

The BALF in the lungs was collected as per Liu et al [33]. In brief, rats were euthanized with intraperitoneal pentobarbital sodium, then the bronchus and lung were exposed. A 3-mm endotracheal cannula was inserted in their trachea. After ligating the hilum of right lung, the left lung was lavaged with 5 mL ice-cold normal saline, which was which retrieved, and the recovery rate was > 90%. The BALF samples were centrifuged (2 000 r/min and 4℃ for 10 min) to pellet the cells. Supernatants were removed and stored at -80℃.

Determination of LDH

A commercial kit was used to determine the amount of LDH release following the manufacturer's protocol. Briefly, the samples were transferred to 96-well plates and incubated at 37 ℃ for 15 min in the presence of 1 mg/ml NADH. Then 2,4-dinitrophenylhydrazine was added to the samples at 37 ℃ for another 15 min. The reaction was stopped by addition of 0.4 M NaOH. Data was determined as the absorbance at 450 nm using a spectrophotometric microplate reader.

Determination of levels of MDA, SOD, GSH, and GSSG

The contents of MDA, SOD, GSH and GSSG in serum were determined according to the Kit commercial instructions.

Western blotting

The experimental procedure of Western blot analysis was carried out as Guo et al [34].Lung tissues were stored at -80 ℃ immediately after rats were sacrificed. Tissue samples (100 mg) were ground with a homogenizer in 1 mL of RIPA lysis buffer with 1mM PMSF and protease inhibitor. Then the homogenate was centrifuged for 20 min at 14 400 r/min at 4 °C to collect supernatant. A bicinchoninic acid (BCA) assay (Thermo Scientifc, MA, USA) was applied to determine the protein concentration. After mixed with loading buffer, the supernatants were heated at 100 °C for 10 min. at a ratio of 1:1 and Equal amounts of the total proteins from each sample were separated by 6%-15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF; EMD Millipore, Burlington, MA, USA). After blocked with 5 % skim milk for 2 h, the blotted membranes were washed with 0.1 % Tween-TBS (TBST), and subsequently incubated with the primary antibodies at 4 ℃ overnight. Then the membranes were washed with TBST buffer three times and incubated with the corresponding secondary antibodies at room temperature for 1 h. After washed with TBST again, the bands were visualized by an enhanced chemiluminescent (ECL) reagent (Thermo Scientifc, MA, USA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All the analyses were assessed using the SPSS 13.0. $P<0.05$ was considered statistically significant.

Results

Histological analysis

First, H&E staining was performed to observe the abnormalities of gross features in the lungs after chlorine exposure under a light microscope. As shown in Figure 1, the rats in the NC group displayed normal appearance and no other histological alteration was observed. In contrast, the lung tissues collected from the group expose to chlorine exhibited marked histopathologic changes, such as alveolar wall thinness, edema, hemorrhage and interstitial infiltration by neutrophils. The airway pathology led to abnormalities in the lung parenchyma with alternating areas of emphysema and atelectasis.

Effect of chlorine on the ROS accumulation

The ROS accumulation was measured by DHE. This probe was oxidized to form intermediate probe-derived radicals that were successively oxidized to generate the corresponding fluorescent products [35]. The results demonstrated that ROS level was significantly increased in chlorine-treated group(Figure 2).

Effect of PTX on the expression of LDH

As LDH release is positively related to cellular damage, the level of LDH was measured to calculate the degree of ALI. It was found that secretion levels of LDH both in serum and BALF were significantly increased following chlorine induction compared with the NC group, while treatments with PTX reduced chlorine induced-LDH increases (Figure 3).

Effect of PTX onof levels of MDA, SOD, GSH and GSSG

To investigate the effect of PTX on oxidative stress, the expressions of biomarkers for oxidative stress, such as MDA, SOD, GSH and GSSG, were detected by a commercial assay kit. It was confirmed that the levels of MDA, SOD and GSSG in chlorine treated rats were up-regulated when compared with the NC group, while the effects of PTX administration were pronounced, except SOD activity. Moreover, expose to chlorine decreased the levels of GSH and GSH/GSSG ratio and administration of PTX to animals remarkably up-regulated these indexes as compared with rats in the model group (Figure 4).

Changes in the protein expression levels of SOD1, SOD2 and CAT

Since regulation of antioxidases may be able to protect against oxidative stress, the present study further investigated whether PTX could affect the expressions of antioxidases. Therefore, the protein expressions levels of SOD 1, SOD 2 and CAT were determined. The western-blot analysis demonstrated that SOD 1 and CAT were markedly up-regulated in the chlorine-treated group compared with the NC group (Figure 5).

Treatment with PTX could inhibit these expressions. Interestingly, chlorine did not affect the expression of SOD2.

Effect of PTX on hypoxia

Since hypoxemia is considered as a significant character of ALI [35], the protein expressions of HIF-1α, VEGF, occludin and E-cadherin were determined. After exposure to chlorine, expressions of HIF-1α, VEGF and occludin were significantly up-regulated in the chlorine group compared to the NC group. Administration of PTX caused a significant decrease in these indicators. In addition, the use of PTX resulted in up-regulated expression of E-cadherin compared to the chlorine group (Figure 6).

Effect of PTX on autophagy

To explore whether the protective effect of PTX on ALI was associated with autophagy activation, we detected the level of several key autophagy-related proteins using western blotting. The results demonstrated that treatment of chlorine significantly down-regulated the ratio of LC3 II/LC3 I and the level of Beclin-1, while Bcl-xl increased, suggesting autophagy activation ($P < 0.05$). PTX could further activate autophagy. To further investigate mitophagy, we searched the expressions of PINK1 and Parkin in the lung tissue. Interestingly, treatment with PTX could promote the expression of PINK1, however, inhabited the level of Parkin in the lung tissue. Because PINK1 selectively accumulates on the surface of damaged mitochondria and initiates the mitophagic process, we examined the expressions of PINK1 and Parkin in the cytoplasm and mitochondria. The results showed that the PINK1 protein expression both in cytoplasm and mitochondria were significantly increased. The Parkin protein expression in cytoplasm increased while decreased in mitochondria (Figure 7).

Discussion

Chlorine is a highly reactive oxidizing toxic gas which is produced globally, such as water purification, bleaching of paper, industrial manufacture of several chemicals, and for many other purposes [7]. Chlorine gas has been used as a chemical weapon since World War I. The easy availability and inherent toxicity make it attractive to aggressors willing to disrupt infrastructure or cause mass panic and casualties. Inhalation of chlorine can produce a range of acute pulmonary effects including impaired lung function, inflammatory reactions, increase of epithelial permeability, and airway hyperresponsiveness [36]. After inhaling chlorine, the features of ALI may be epithelial cell death, inflammation, pulmonary edema, hypoxemia, and pulmonary function abnormalities, which are key aspects in animal models and human clinical studies [37]. In present chlorine-exposed rat model, we observed epithelial damage, alveolar injury and inflammation, which agreed with previous studies in several animal models [38–39]. We also noticed pulmonary edema and ROS accumulation 3 h after chlorine exposure. These data from this study combined with our previous findings clearly suggested that our rat model for chlorine-induced ALI is produced successfully.

Currently, anti-inflammatory drugs remain an effective therapy of ALI. Treatment with glucocorticoids, such as dexamethasone led to significant improvement of lung functions and to reduced inflammation [40]. In clinical use for 30 past years, PTX has been licensed for use in peripheral vascular disease. It may increase the deformability of erythrocytes, reduce blood viscosity, and inhibit fibrotic progression [41–42]. Chavarria et al. found that pentoxifylline might be a standard therapy to improve prognosis of patients with pneumonia by COVID-19 with antioxidants [43]. In the present study, we found that PTX treatment ameliorated the level of the LDH both in BALF and serum effectively, indicating the protective effect of PTX in lung injury induced by chlorine.

Due to oxidative stress plays a crucial role in the development of ALI [14], we measured oxidative stress markers. As an indicator of lipid peroxidation, MDA is produced in oxidative cellular damage which indicates that ROS is overproduced [44]. PTX treatment caused a significant decrease in lung tissue levels of MDA. To further investigate related mechanisms, we determined antioxidant enzymes and antioxidants. SOD is one of the major intracellular antioxidant enzymes that induces superoxide anions (O^{2-}) free radical to hydrogen peroxide (H_2O_2). Then, H_2O_2 can be reduced by converting to H_2O in the presence of CAT [45]. As one of the nature antioxidants, GSH plays important roles in reducing the tissues from damage via detoxifying electrophiles, scavenging ROS, maintaining the essential thiol status of proteins, and providing a reservoir for cysteine. During ROS formation, GSH is converted to GSH disulfide (GSSG). In the current study, PTX also suppressed the levels of SOD1, GSSG and expression of CAT, and enhanced the GSH level and GSH/GSSG ratio to protect against pulmonary injury. Therefore, we suggested that PTX has a beneficial antioxidative effect on ALI induced by chlorine.

Hypoxia is closely related to oxidative stress in inflammatory lung diseases [46]. In response to hypoxia, the tissue induces the expression of HIF-1, which is widely expressed. As a transcriptional heterodimer, HIF-1exerts a vital pathophysiological role in oxygen homeostasis [47]. Jahani et al. considered that hypoxia might be a key feature of COVID-19 launching activation of HIF-1 [48]. Under hypoxic conditions, ROS released from the mitochondrial electron transport chain can participate in the regulation of HIF-1 activity [49]. In this study, we showed that PTX directly reversed overexpression of HIF-1α. The classical HIF-1α/VEGF signaling pathway also exerts an important role in the pathogeneses of ALI and pulmonary edema. In addition, HIF-1α induces and activates the overexpression of the VEGF gene, which consequently affects the expression of tight junction proteins and adhesion molecules [50–51]. The present study found that PTX significantly inhibited the overexpression of VEGF and occludin accompanied by the upregulation of E-cadherin, which were in agreement with previous researches [52–53]. These findings showed that the ROS/HIF-1α/VEGF signaling pathway in the lung tissues of rat models in chlorine-induced ALI was activated.

Autophagy, a major cellular defense against oxidative stress, is an intracellular digestion system that works as an inducible adaptive response to ALI. ROS may activate autophagy, and then facilitate cellular adaptation and diminish the damaged macromolecules and dysfunctional organelles [54]. Numerous regulators like LC3 II and Beclin 1 play important role in process of autophagy induction during lung injury. After binding to the lipid derivative phosphatidylethanolamine, LC 3 I is converted to form LC 3 II,

which enables fusion with the lysosomes. In addition, the ratio of LC 3 I/LC 3 II is used as an indicator of autophagy. As a is part of a Class III PI3K complex, the Beclin 1 takes part in autophagosome formation though assembling around cargo in a vesicle and combining with lysosome[55]. As our results demonstrated, PTX enhanced the expression of LC3 II and Beclin 1 accompanied by the reducing of Bcl-xl, suggesting that autophagy exerted a protective role in ALI induced by chlorine.

Because mitochondria are considered as the main contributor of reactive oxygen species, the removal of damaged mitochondria by mitophagy plays important role in cellular antioxidant defenses [56]. PINK1, as a mitochondrially targeted serine–threonine kinase, takes part in mitochondrial quality control. Under normal conditions, PINK 1 maintains low basal levels though importing into the mitochondrial intermembrane space and rapidly degraded when combined with the presenilin-associated rhomboid-like protein (PARL) and the proteasome. When mitochondria are depolarization, PINK1 accumulates on the mitochondrial outer membrane (OMM) and results in recruitment of Parkin from the cytosol, then activates mitophagy [57]. Subsequently, the expressions of PINK1 and Parkin were performed to investigate the potential mechanism. Interestingly, PTX had been shown to inhibit the expression of PINK1, but increased the expression of Parkin. After separating mitochondria and cytoplasm, we found that the expression of PINK1 and Parkin in mitochondria showed similar trends with these expression in lung tissues. However, PTX treatment reduced the expression of PINK1 and Parkin in cytoplasm compared to the chlorine group. These results showed that PTX exerted a protective role in attenuating ALI induced by chlorine through improving autophagy, especially mitophagy.

In conclusion, PTX might be a potential therapy option for ALI induced by chlorine. In the present study, PTX attenuated chlorine induced ALI via regulating oxidative stress, hypoxia and autophagy. However, more research will be needed to explore specific mechanism.

Declarations

Acknowledgements

Not applicable

Authors' contributions

MM Liu and JZ Liu wrote the main manuscript text; MM Liu and CQ Zhao prepared figures 1-2; MM Liu, JZ Liu P Guo, R Liu, H Wu, WH Yu, Z Wang, and CX Hai prepared figures 3-7. XD Zhang, MM Liu and JZ Liu designed the investigation. All authors reviewed the manuscript.

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Availability of data and materials

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

Ethics approval and consent to participate

All experimental studies as approved by the ethics committee of Fourth Military Medical University' Animal Care and Use Committee, all animals' experiments were approved by science faculty and all are based on the approved guidelines and are by ARRIVE (Animal Research) Reporting of In vivo Experiments guideline ((permit no. IACUC-20190320)).

Consent for publication

Not applicable.

Competing interests

The authors report no conflict of interest.

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Figures

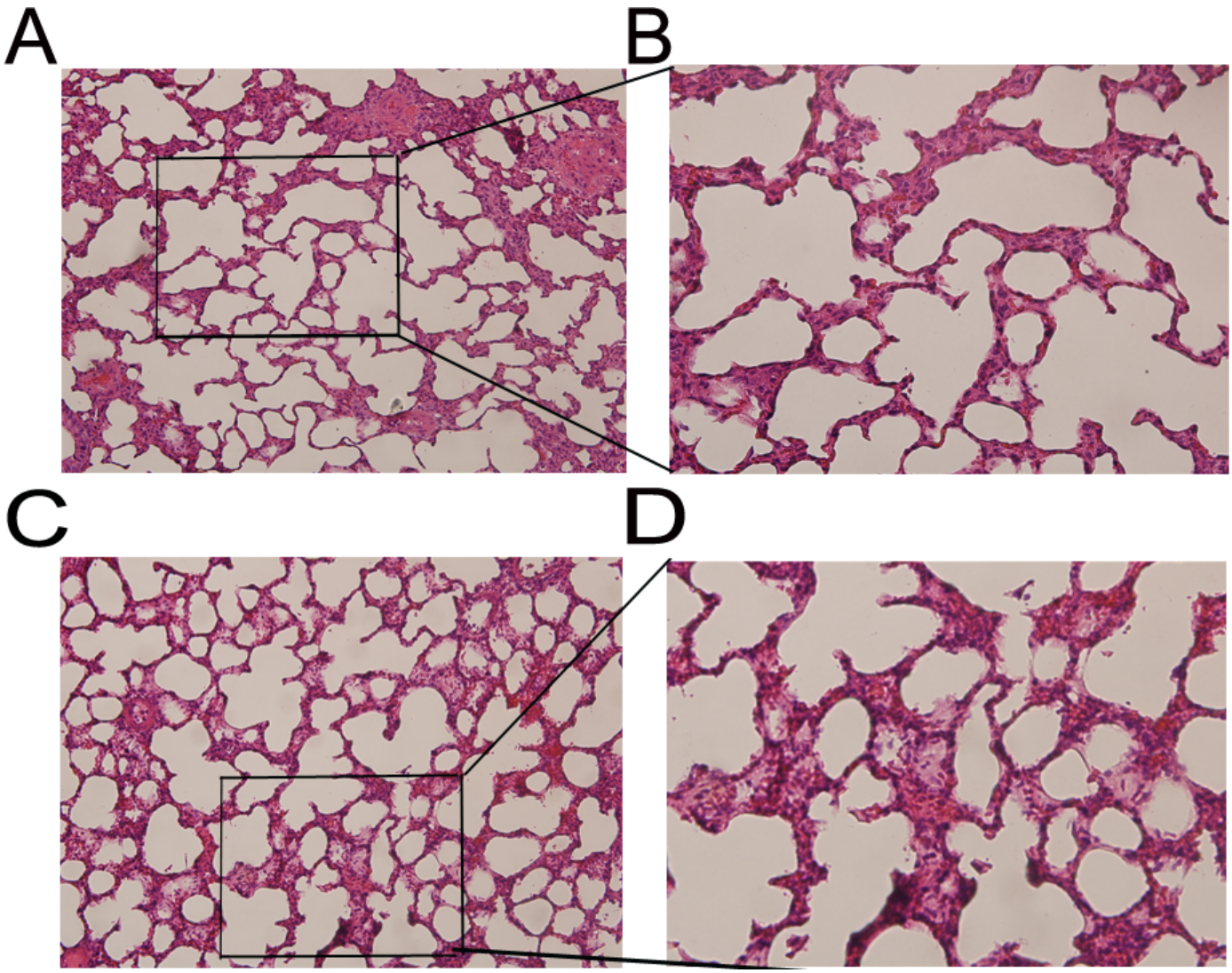

Figure 1

Pathologic changes in rats exposed to chlorine. (A) H&E staining in the lungs of rats in NC group (× 200); (B) H&E staining in the lungs of rats in NC group (× 400); (C) H&E staining in the lungs of rats in chlorine group (× 200); (D) H&E staining in the lungs of rats in chlorine group (× 400). H&E: hematoxylin and eosin; NC: normal control.

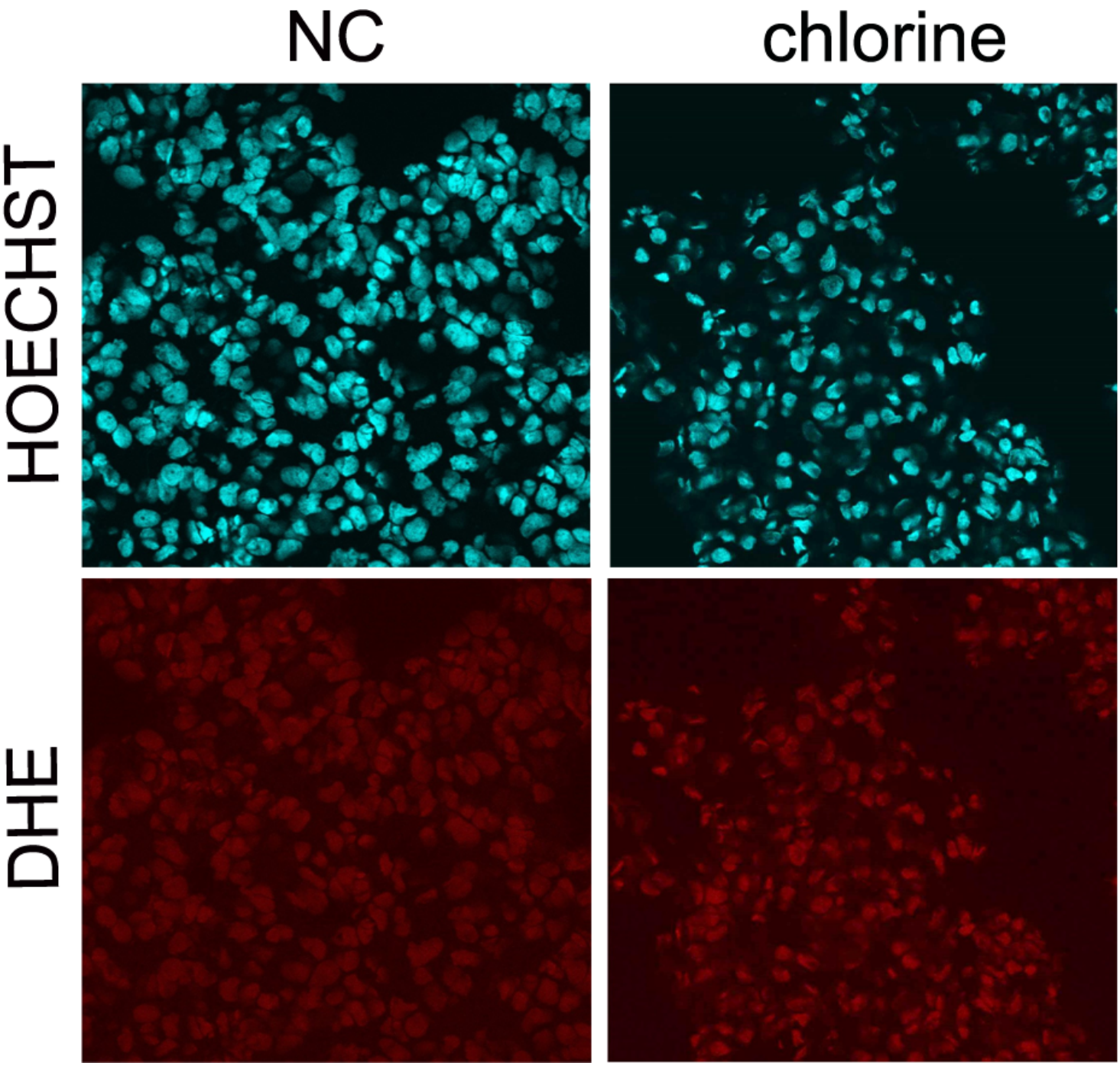

Figure 2

ROS accumulation in rats exposed to chlorine (× 600). NC: normal control.

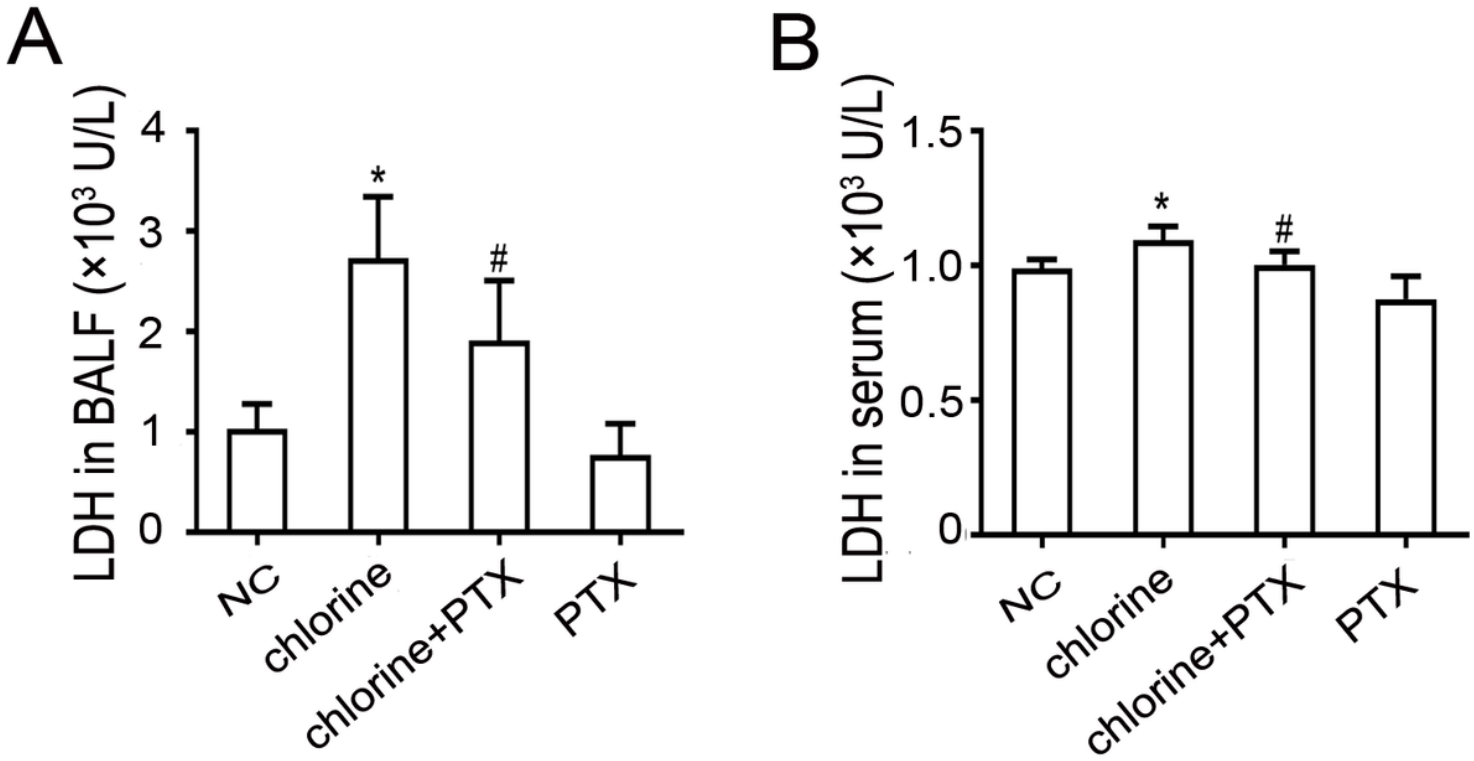

Figure 3

The effect of PTX on the level of LDH. The content of LDH in BALF (A) and serum (B). Data are presented as mean ± S.D. (n = 6). * $P < 0.05$ compared with the control group. #$P < 0.05$ compared with chlorine-treated group.NC: normal control; LDH: lactic dehydrogenase; BALF: Bronchoalveolar lavage fluid; PTX: pentoxifylline.

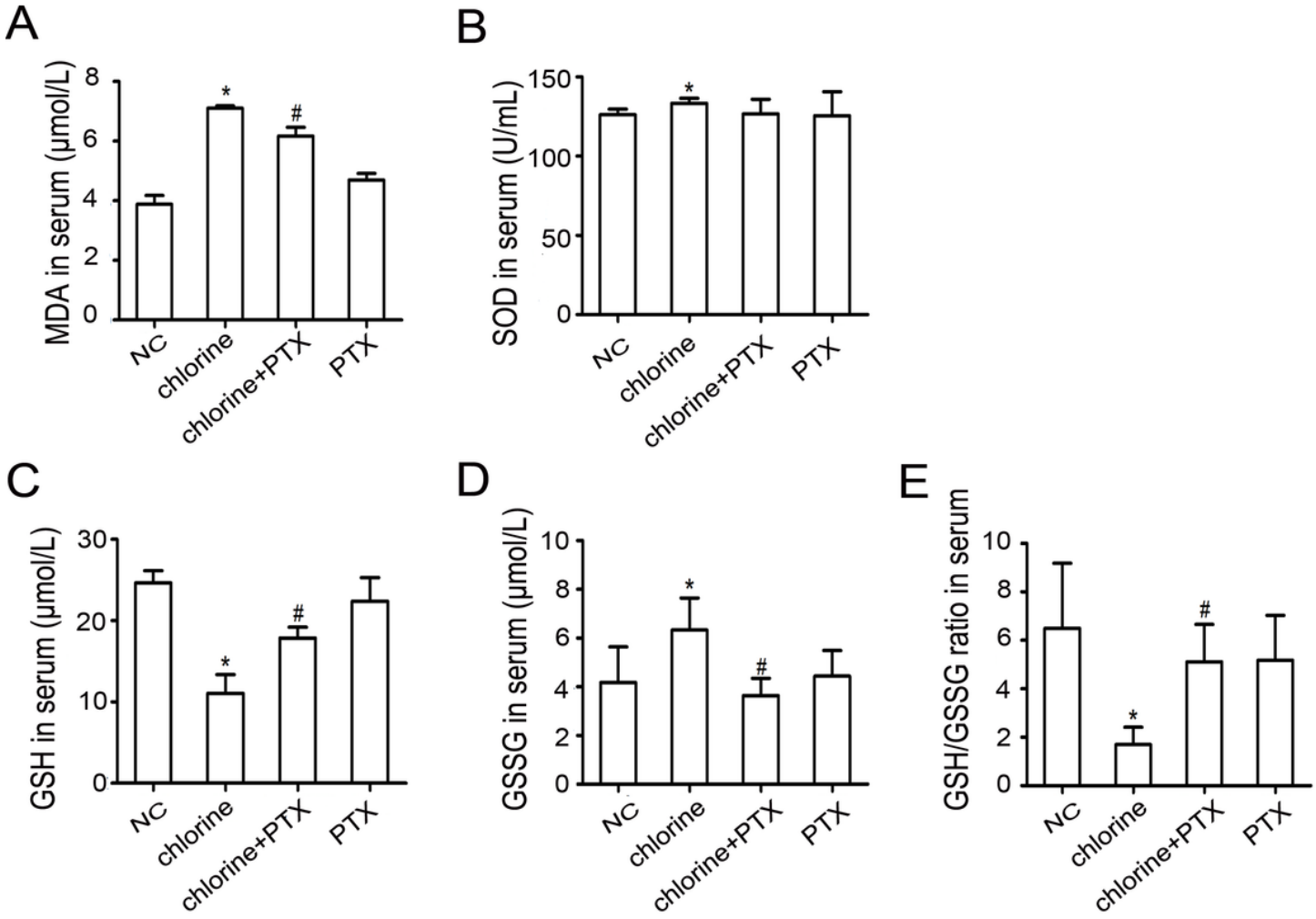

Figure 4

The effect of PTX on the content of MDA, SOD, GSH, GSSG and GSH/GSSG ratio. (A) The content of MDA, (B) the level of SOD, (C) the content of GSH, (D) the content of GSSG and (E) GSH/GSSG ratio. * $P <$ 0.05 compared with the control group. #$P <$ 0.05 compared with chlorine-treated group. NC: normal control; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: Glutathione; GSSG: oxidized glutathione; PTX: pentoxifylline.

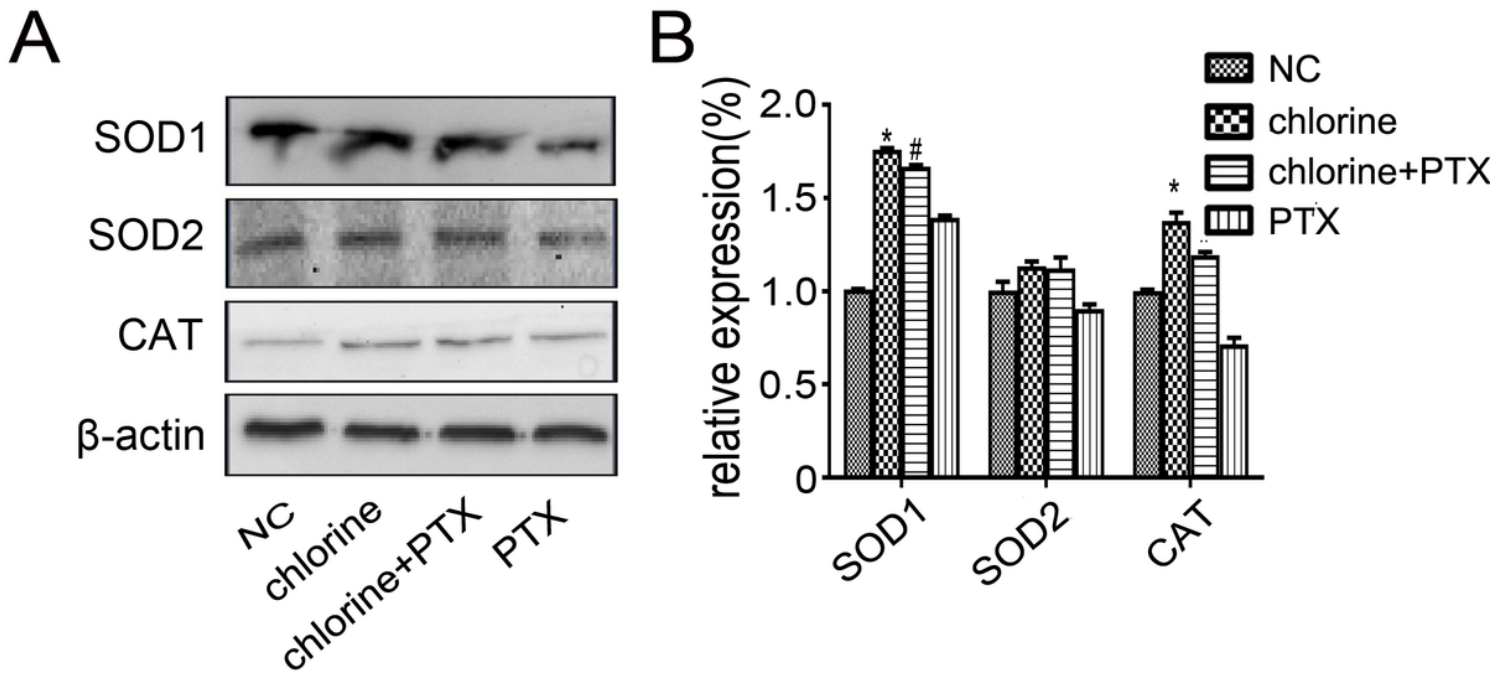

Figure 5

Effect of PTX on SOD1, SOD2 and CAT protein expression in rat lung tissue following chlorine exposure. (A) The protein expression levels were determined by western blot analysis. (B) Densitometric analyses of protein expression levels corresponding to (A). * $P < 0.05$ compared with the control group. #$P < 0.05$ compared with chlorine-treated group. NC: normal control; PTX: pentoxifylline; MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase.

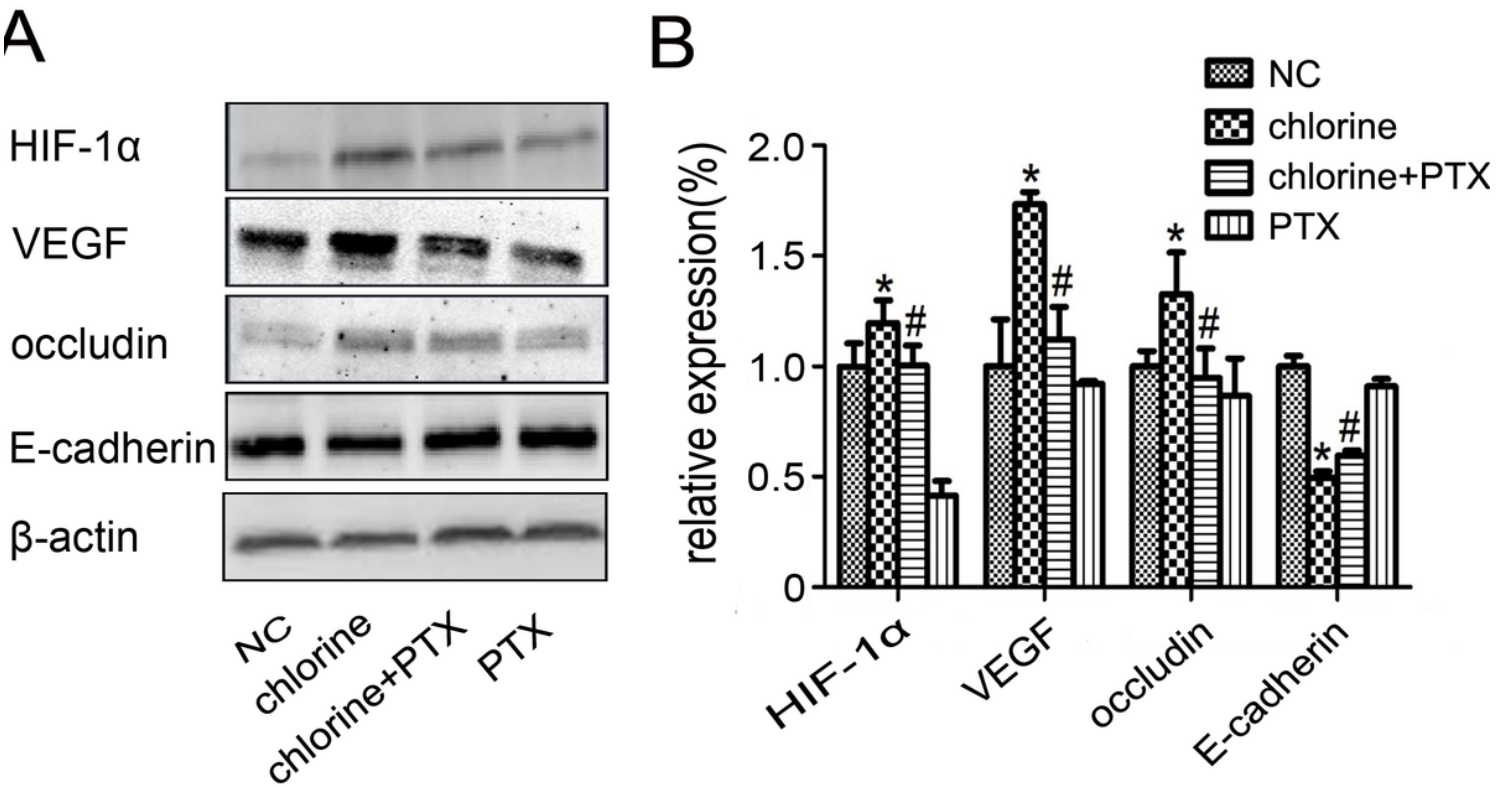

Figure 6

Effect of PTX on HIF-1α/VEGF signaling pathway in ALI induced by chlorine. (A) The protein expression levels were determined by western blot analysis. (B) Densitometric analyses of protein expression levels corresponding to (A). * $P < 0.05$ compared with the control group. #$P < 0.05$ compared with chlorine-treated group. NC: normal control; PTX: pentoxifylline; HIF-1α: Hypoxia-Inducible Factor-1α.

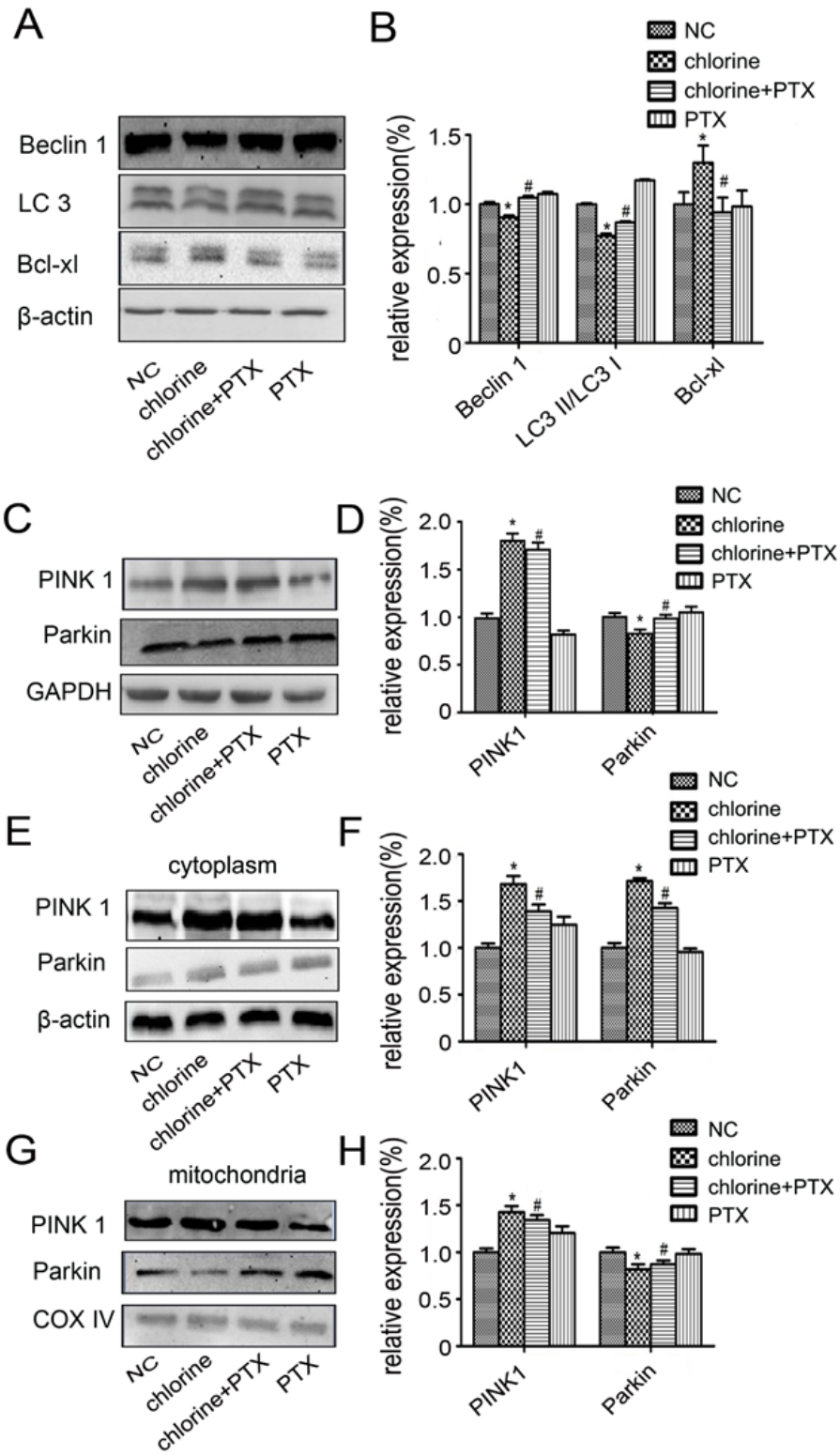

Figure 7

Effect of PTX on autophagy in ALI induced by chlorine. (A, C, E and G) The protein expression levels were determined by western blot analysis. (B, D, F and H) Densitometric analyses of protein expression levels corresponding to (A, C, E and G) respectively. * $P < 0.05$ compared with the control group. #$P < 0.05$ compared with chlorine-treated group. NC: normal control; PTX: pentoxifylline; PINK1: PTEN Induced Putative Kinase 1.