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Heme oxygenase-1 attenuates seawater drowning-induced acute lung injury through a reduction in inflammation and oxidative stress

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ABSTRACT

Objective: Heme oxygenase-1 (HO-1) plays a critical protective role in various insults-induced acute lung injury (ALI) through its strong anti-inflammatory, anti-oxidant, and anti-apoptotic properties, but its protective role and mechanism on seawater aspiration-induced acute lung injury remains unclear. This study aimed to explore the therapeutic potential and mechanism of HO-1 to attenuate seawater aspiration-induced ALI in vivo and in vitro.

Methods: The viability and invasion of A549 cell were analyzed through cell counting kit-8 and lactate dehydrogenase release assay; the transcriptional level of inflammatory cytokines (TNF-α, IL-6, IL-8 and MCP-1) and cell proliferation-related cytokines (FoxM1, Ccnb1 and Cdc25C) in seawater-treated A549 cell were tested by qPCR; apoptotic cells were analyzed by flow cytometryd; HO-1mRNA and protein were determined by qPCR and western blotting; the fluorescent indicators (DCFH-DA, dihydroethidium, MitoSox Red and Fluo-4) were used to monitor generation of ROS and mitochondrial function. The lung wet/dry weight radio and lactate dehydrogenase activity, Sirius red staining, TUNEL assay and immunohistochemical staining with anti-pan Cytokeratin antibody were analyzed in seawater-drowning mice. The role of HO-1 on seawater-drowning pulmonary injury was explored via HO-1 activity inhibitors (Zinc protoporphyrin) in vitro *and* in vivo.

Results: Seawater exposure decreased the cellular viability, increased the production of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α), induced cellular apoptosis and inhibited the expression of cell proliferation-related cytokines (FoxM1, Ccnb1 and Cdc25C). Moreover, seawater exposure led to mitochondrial dysfunction in A549 cells. Supplement of HO-1 sepcific inducer (heme) or its catalytic product (biliverdin) significantly attenuated seawater-induced A549 damage and promoted cell proliferation. However, Zinc protoporphyrin abolished the beneficial effects of HO-1 on seawater drowning-induced pulmonary tissue injury.

Conclusion: HO-1 attenuates seawater drowning-induced lung injury by its anti-inflammatory, anti-oxidative, and anti-apoptosis function.

1. Introduction

Drowning is the second accidental cause of death in the world [1,2]. The World Health Organization estimates that drowning accounts for approximately 450,000 deaths each year [3]. Approximately 1/3 of near-drowning patients suffer from severe acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [4]. Seawater with high osmolarity directly damages to alveolar epithelial cells, leads to fetal hypoxia and acidosis, and thereby eventually causes ALI/ARDS. In drowning patients, seawater inhalation results in lung oxidative stress, pneumonia, hemorrhage, alveolar epithelial cell necrosis and atelectasis [5,6]. Although the pathophysiological and molecular mechanisms have been investigated, little is known about the crucial cellular and molecular mechanisms of seawater-induced ALI.

The recent studies have shown that free hemoglobin and heme levels increase in alveolar space from ARDS patients [7]. Free heme in lung deteriorated oxidative stress injury and toll-like receptor-dependent inflammation [8]. Heme oxygenase-1 (HO-1) is a rate-limiting enzyme of heme degradation, which catalyzes the degradation of free heme to biliverdin (BV), ferrous iron and carbon monoxide. HO-1 and catalytic products (BV and carbon monoxide), have been proved to possess potent antioxidant activity and anti-inflammatory effects in

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response to many harmful stimuli such as hypoxia, oxidative stress and radiation [9,10]. Exogenous BV supplement could suppress the aggregation of white blood cells, led to reduction in generation of pro-inflammatory cytokines and proteins, ultimately attenuated inflammation [11–13]. Furthermore, Nrf-2/HO-1 pathway participated in the protective role of hydrogen gas in seawater instillation-induced ALI in rabbits [14]. The expression and activities of HO-1 significantly up-regulated on seawater drowning-induced ALI in mice [15]. However, the role of HO-1 is unclear in seawater drowning-induced ALI. In this study, we investigated the roles and detailed mechanisms of HO-1 in attenuating seawater-induced ALI in vivo and in vitro.

2. Materials and methods

2.1. Reagents and antibodies

Seawater was prepared according to the major compositions of the East China Sea provided by the Chinese Ocean Bureau (osmolality 1300 mmol/L, pH 8.2, specific weight 1.05, NaCl 26.518 g/L, $MgSO_4$ 3.305 g/L, $MgCl_2$ 2.447 g/L, $CaCl_2$ 1.141 g/L, KCl 0.725 g/L, and $NaHCO_3$ 0.202 g/L) [16]. Biliverdin hydrochloride was purchased from Frontier Scientific (Logan, USA). Hemin, zinc protoporphyrin IX (ZnPP), dimethyl sulfoxide (DMSO), dihydroethidium (DHE) and rhodamine 123 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Biosharp Life Sciences (Biosharp, China). In Situ Cell Death Detection Kit was purchased from Boster Biological Technology. Primary antibodies for anti-HO-1 (Cat # ab13248), anti-GAPDH (Cat # ab9485) and anti-pan Cytokeratin (Cat # ab7753) were obtained from Abcam (Abcam, USA).

2.2. Cell culture and treatment

Human lung epithelial cell line (A549, obtained from the Shanghai Institute of Cell Biology, Shanghai, China), was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin, 100 U/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were exposed to culture medium without compound as control group, and to culture medium with 25% seawater (0.25 ml per 1 mL total volume) for 6 h as seawater (SW) group. Hemin (10 μM) treated for 18 h before exposed to seawater. BV (15 μM) was treated for 24 h before exposed to seawater [17]. ZnPP (10 μM) was treated for 1 h before hemin administration [18].

2.3. Cell viability assay

Cells viability was measured using Cell Counting Kit-8 (CCK-8) kits. Briefly, cells were washed with phosphate buffer (PBS) and incubated for an additional 2 h with CCK-8 reagent (100 μL/ml medium) at 37 °C. The absorption values were measured at 450 nm using a microplate reader (BioTek, Winooski, USA). Additionally, the release of lactate dehydrogenase was assessed by using a commercial kit (Nanjing Jiancheng Bio Co., Ltd., China) according to the manufacturer's protocol and expressed as international units per liter (U/L).

2.4. Reverse transcription-quantitative polymerase chain reaction

Messenger RNA expression was measured by quantitative polymerase chain reaction (qPCR) with specific primer sequences and the system protocol SYBR Premix Ex Taq (Takara, Japan). All the primers in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and listed in Table 1. The $2^{-\Delta\Delta Ct}$ method was used to quantify the gene expression [19].

2.5. Western blot

Protein expression studies were performed as previously described [20]. Briefly, the samples were homogenized in ice-cold RIPA buffer with protease and phosphatase inhibitor mixture (Roche Diagnostics) for 30 min. The supernatant was collected after centrifugation (12,000 rpm, 10 min and 4 °C). Equal protein concentration was measured by standard procedures. Antibodies used for Western blot analysis were anti-HO-1 (diluted 1:1000), anti-GAPDH (diluted 1:10,000). After washing with PBS four times, the blots were incubated with secondary antibodies (diluted 1:5000, Boster) for 2 h at room temperature. The protein bands was determined and standardized by a BeyoECL Star (Beyotime).

2.6. Flow cytometry

The percentage of apoptotic cells was determined using Annexin V-FITC/PI Apoptosis Detection Kit (Beijing Cowin Biotech Co., Ltd., China) according to the manufacturer's instruction through flow cytometry (BD Biosciences, C6 Plus, USA).

2.7. HO-1 activity

The catalytic activity of HO-1 was calculated by the spectrophotometric determination of bilirubin production as described previously [21]. Final reaction concentrations were: 25 μM hemin (Sigma-Aldrich, Cat #51280), 1 mM β-NADPH (Sigma-Aldrich, Cat #N5130), 2 U cytochrome P450 reductase (Sigma-Aldrich, Cat #C8113), 0.25 mg/ml protein, 5 mM deferoxamine mesylate salt and 2 mg/mL partially purified rat liver biliverdin reductase preparation. The reactions were incubated for 60 min in a 37 °C water bath in the dark. The reactions were terminated by addition of the same volumes of chloroform. The concentration of bilirubin was spectrophotometrically determined by measuring the difference in absorbance between 465 and 530 nm, with a molar extinction coefficient of 40/mM/cm.

2.8. Detection of intracellular ROS by fluorometric intracellular ROS kit

Reactive oxygen species (ROS) was monitored using a Fluorometric Intracellular ROS kit (Nanjing Jiancheng Bio Co., Ltd., China). Briefly, A549 were treated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) for 60 min in the dark, washed with PBS. The fluorescence intensity was detected by fluorescence spectrophotometer (wavelength was 485 nm and the emission wavelength was 530 nm).

2.9. Detection of intracellular ROS by dihydroethidium (DHE) and MitoSox Red fluorescent probes

A549 cells were treated with DHE (10 μM) for 30 min or MitoSox Red (5 μM) for 10 min and washed with PBS. The images were collected using Nikon TE-2000 fluorescence microscope (Nikon, Tokyo, Japan).

2.10. Measurement of intracellular calcium flux by Fluo-4 probes

The intracellular calcium flux ($[Ca^{2+}]_i$) was measured using Fluo-4 (5 μM) which was combined with Hoechst 33342 (1 μg/ml). Cellular fluorescence images were acquired by using Nikon TE-2000 fluorescence microscope (Nikon, Tokyo, Japan).

2.11. Mitochondrial membrane potential (MMP) assay

Mitochondrial membrane potential (MMP) was detected using fluorescent dyes (5 μM rhodamine 123 and 5 μM JC-1). The fluorescence intensity by rhodamine 123 staining was measured with a microplate reader using 485 nm excitation and 529 nm emission filter settings; and cellular fluorescence images were acquired using Nikon TE-2000 fluorescence microscope (Nikon, Tokyo, Japan).

Table 1
Primers used in this study for PCR.

Gene	Forward	Reverse
Tnf	5′-CTCCTCACCCACACCATCAGCCGCA-3′	5′-ATAGATGGGCTCATACCAGGGCTTG-3′
Il6	5′-GATGAGTACAAAAGTCCTGATCCA-3′	5′-CTGCAGCCACTGGTTCTGT-3′
Il8	5′-CTCTCTTGGCAGCCTTCCTGA-3′	5′-CCCTCTGCACCCAGTTTTCCTT-3′
Ccl2	5′-CTCTGCCGCCCTTCTGTG-3′	5′-TGCATCTGGCTGAGCGAG-3′
FoxM1	5′-ATAGCAAGCGAGTCCGCATT-3′	5′-AGCAGCACTGATAAACAAAGAAAGA-3′
Ccnb1	5′-AATAAGGCGAAGATCAACATGGC	5′-TTTGTTACCAATGTCCCCAAGAG-3′
Cdc25C	5′-TCTACGGAACTCTTCTCATCCAC-3′	5′-TCCAGGAGCAGGTTTAACATTTT-3′
Gapdh	5′-TCTCCTCTGACTTCAACAGCGAC-3′	5′-CCCTGTTGCTGTAGCCAAATTC-3′

2.12. Seawater drowning model and animal treatments

BALB/c mice (male, weighted 18-25 g) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animals were allowed to acclimate to the appropriate environment for 7 days. All experiments were performed in accordance with relevant laws and institutional guidelines, and approved by the Ethics Committee for the Use of Experimental Animals in Jiangnan University.

Acute lung injury model induced by seawater drowning in mice was performed as previously described [15]. Briefly, mice were packed into the mice holder and immersed into a water tank containing 6 cm depth and 25 ± 2 °C temperature seawater for 28 s. Mice randomly divided into the following groups ($n = 6$): control group, ZnPP groups (7 days and 15 days), seawater drowning groups (3 days, 7 days, 15 days after drowning) and seawater drowning (7 days and 15 days after drowning) + ZnPP groups. ZnPP groups and seawater drowning + ZnPP groups daily treated intraperitoneally with ZnPP (50 μmol/kg) [20]. Finally, mice were sacrificed and lung tissue samples were collected and evaluated.

2.13. Tissue collection and histological analysis

Right lungs were collected for determined the lung W/D ratio, biochemical analysis and western blot. Left lungs were fixed with 4% paraformaldehyde for histological examination. Tissue was cut into 4 μm thick sections for subsequent histological analysis. The severity of lung injury was evaluated staining with hematoxylin & eosin and lung injury scores [22]. Fibrotic area was evaluated on lung tissue sections stained with sirius red staining, and fibrosis was expressed as percentage of stained area over total lung tissue. TUNEL assay and immumohistochemical staining with anti-pan Cytokeratin antibody was tested in lung tissue sections. All sections were observed under microscopy (LSM 510 confocal laser scanning microscopy, Zeiss).

2.14. Statistical analysis

All experiments were performed at least thrice with similar results. Data were expressed as means ± standard deviation (SD). Comparisons between groups were carried out by analysis of variance (ANOVA) with a SPSS package. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Seawater induced A549 cell damage and inhibited cellular proliferation

The cellular morphology of A549 cell with seawater exposure (0–24 h) was observed by bright-field microscope. The cell damage was analyzed by CCK-8 assay and LDH release assay. The results showed that seawater exposure changed A549 morphology (Fig. 1A) and decreased cellular viability in time-dependent manner (Fig. 1B and C). To investigate whether seawater had an effect on inflammatory cytokines in A549 cell, we detected the mRNA level of inflammatory cytokines (TNF-α, IL-6, IL-8 and MCP-1) by qPCR. The results revealed that seawater could enhance the production of these pro-inflammatory cytokines in A549 cell (Fig. 1D). To evaluate the effect of seawater on cell apoptosis, we detected apoptotic changes by flow cytometry in seawater-treated A549 cells and found that seawater led to A549 cells apoptosis (Fig. 1E). Then we measured the expression changes of cell proliferation- related cytokines (FoxM1, Ccnb1 and Cdc25C) in seawater-treated A549 cell. The cell proliferation assays showed that seawater inhibited the expression of cell proliferation-related cytokines in A549 cell (Fig. 1F). Furthermore, seawater stimuli did not increase the expression and catalytic activity of HO-1 (Fig. 1G–H) when HO-1 inducer (heme) were absent. These data suggested that seawater exposure directly induced cell damage and inhibited cell proliferation in A549 cell. Considering that 25% seawater (0.25 ml per 1 ml total volume) at 6 h had significant damages in A549 cell, the test condition was chosen for following studies to investigate the roles and mechanisms of HO-1 in attenuating seawater-induced cellular damage in vitro.

3.2. HO-1 and biliverdin attenuated seawater-induced cell damage and promoted cell proliferation in A549 cell

To investigate the cytoprotective effect of HO-1 in seawater-treated A549 cell, we pretreated cell with hemin (a potent HO-1 inducer, 10 μM), or biliverdin (BV) (a catalytic product of HO-1, 15 μM) and ZnPP (a HO-1 activity inhibitor, 10 μM). As shown in Fig. 2A–B, hemin administration significantly enhanced HO-1 protein expression and activity compared with the control group and seawater group. ZnPP could significantly inhibit the catalytic activity of HO-1 in seawater + hemin + ZnPP group when compared with the seawater + hemin group. The cell viability was enhanced by hemin and BV while ZnPP could abrogate the protective effect of hemin in seawater-treated A549 cell (Fig. 2C). Luckily, BV could reverse the harmful effect of ZnPP on cell viability in seawater + hemin + ZnPP + BV group. The production of pro-inflammatory cytokines (TNF-α, IL-6 and IL-8) was decreased by hemin and BV in seawater-treated A549 cell, while ZnPP increased these cytokines production in seawater + hemin + ZnPP group (Fig. 2D). BV could abrogate the effect of ZnPP in seawater + hemin + ZnPP + BV group. In cell proliferation experiments, as shown in Fig. 2E–F, the results showed that hemin and BV could inhibit cell apoptosis and increase the expression of cell proliferation-related cytokines (FoxM1 and Ccnb1) in seawater-treated A549 cell. The effect of hemin was abrogated by ZnPP in seawater + hemin + ZnPP group, and ZnPP had no effect on BV treatment in seawater + hemin + ZnPP + BV group. In summary, these data suggested that HO-1 and its catalytic product (biliverdin) could attenuate seawater-induced cell injury and promote cell proliferation in A549 cell.

3.3. HO-1 and biliverdin protected cells by reducing ROS generation and attenuating mitochondrial damage

The recent studies have shown that increased ROS play an important role in the pathogenesis of SW-induced ALI [23]. Therefore, we hypothesized that the cytoprotective effects of HO-1 and its catalytic

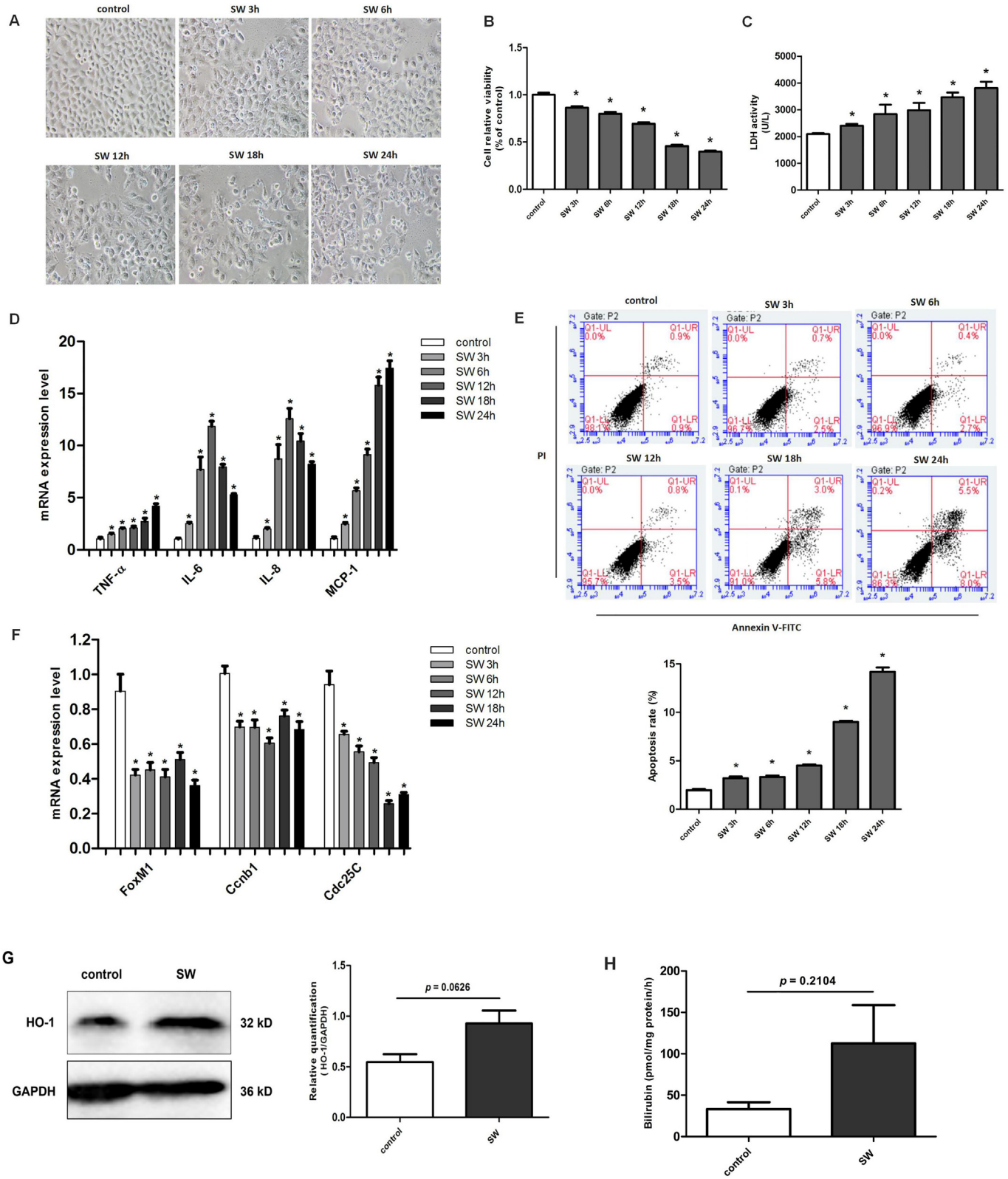


Fig. 1. Seawater exposure induced cell damage and inhibits cell proliferation of A549 cell. A549 cells were treated with 25% seawater in various times (A–F). A. Representative cell morphology images. Scale bars, 50 μm. B. CCK-8 assay for the determination of cell viability. C. The cytotoxicity was measured by LDH release assay. D. The relative mRNA levels of the inflammatory cytokines were assessed by qPCR. E. Apoptosis was analyzed using Annexin V-FITC/PI staining with flow cytometry. The columnar picture is the statistical analysis of the apoptosis rate. F. The relative mRNA levels of cell proliferation-related cytokines were detected by qPCR. A549 cells were treated with 25% seawater for 6 h (G and H). G. Representative western blot images and the analysis to quantify HO-1 levels. H. HO-1 activities. The data are expressed as the mean ± SD (n = 5 for each group). *P < 0.05 vs. control group.

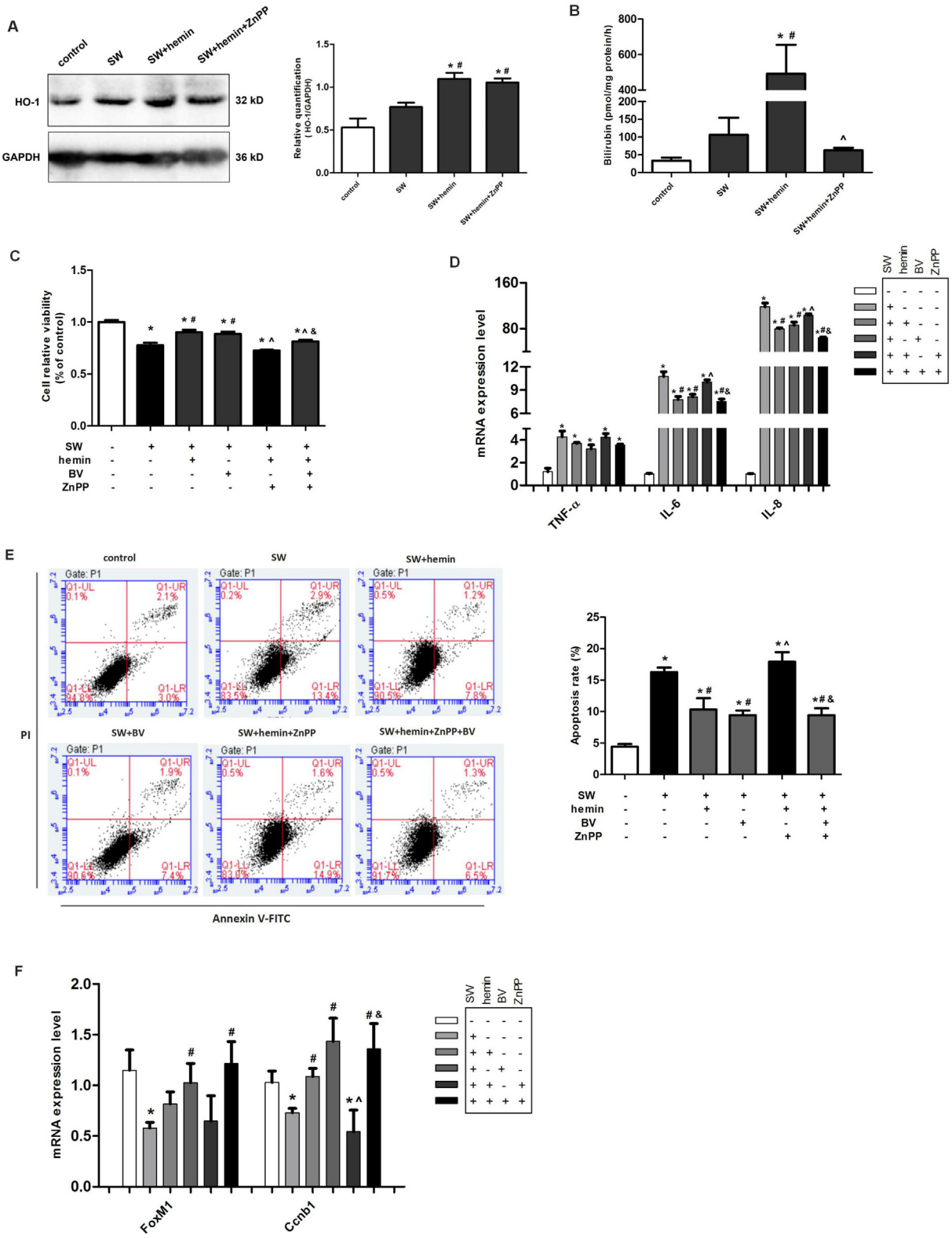
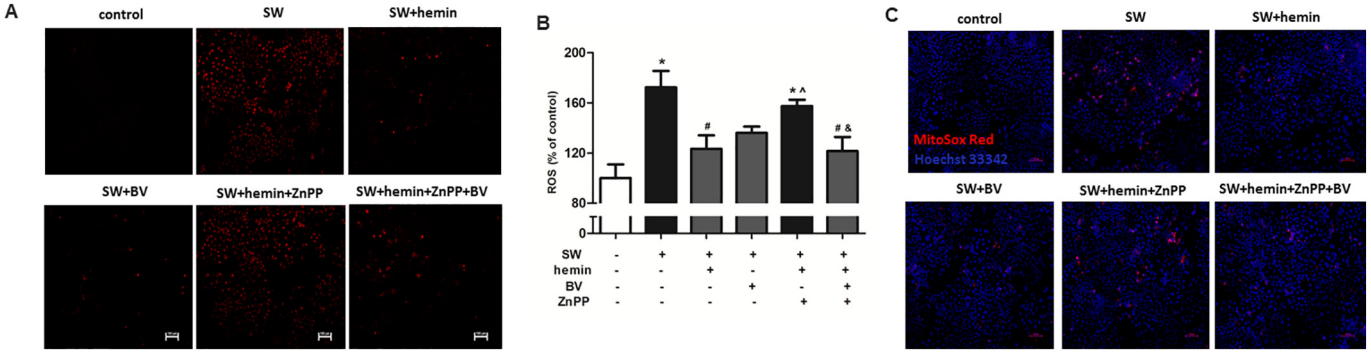
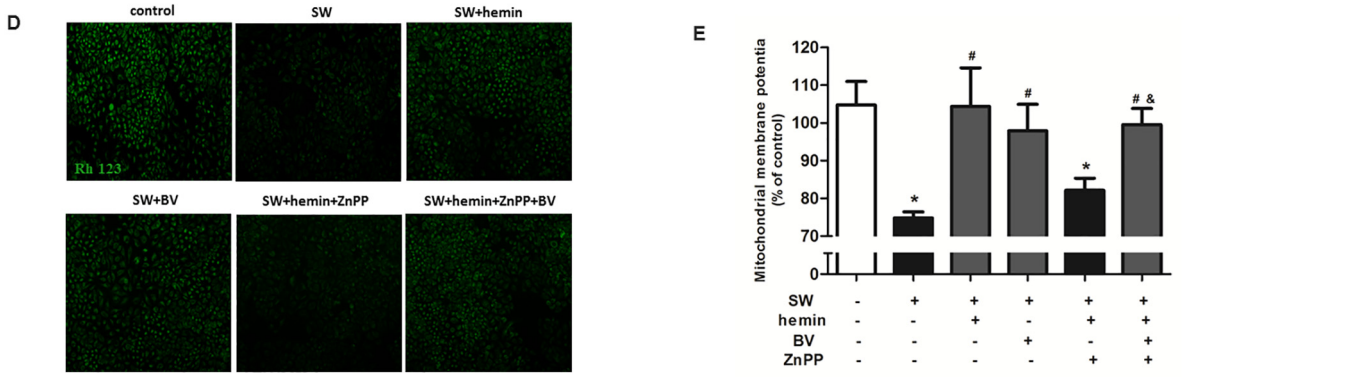
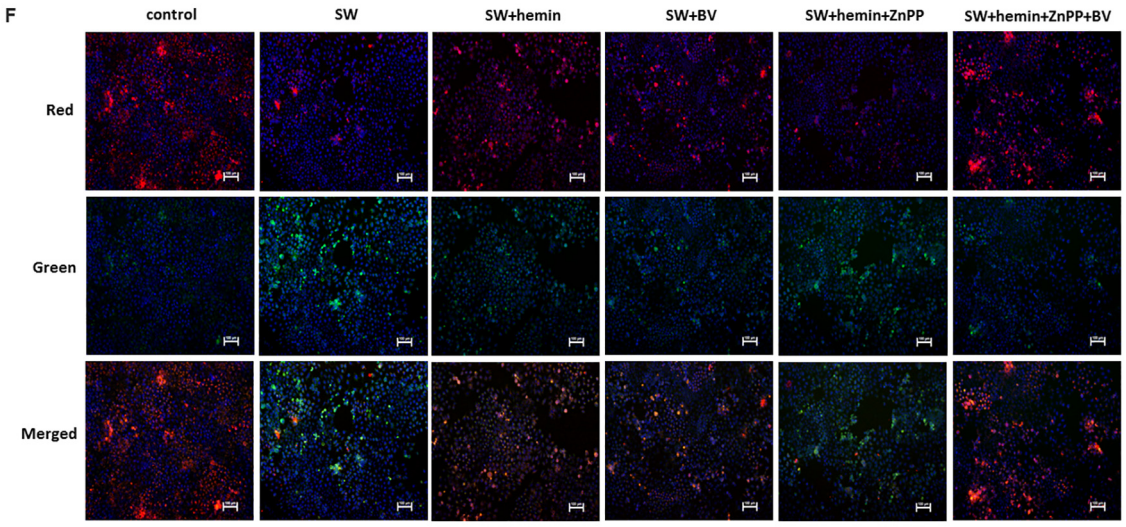


Fig. 2. HO-1 and BV attenuated cell damage and promoted cell proliferation in seawater-treated A549 cell. A. Representative western blot images and the analysis to quantify HO-1 levels. B. HO-1 activity. C. The cell viability was measured by CCK-8 assay. D. The relative mRNA levels of the inflammatory cytokines were assessed by qPCR. E. Apoptosis was detected using Annexin V-FITC/PI staining with flow cytometry. The columnar picture is the quantitative result of apoptosis. F. The expression of cell proliferation-related cytokines were determined by qPCR. Values represented mean ± SD (n = 5 for each group). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. SW group; $^{\wedge}P < 0.05$ vs. hemin + SW group; $^{\&}P < 0.05$ vs. hemin + ZnPP + SW group.

product depend on the reduction of ROS production and the attenuation of mitochondrial damage in seawater-treated cell. To measure the effect of HO-1 and its catalytic product on the ROS generation induced by seawater and mitochondrial function changes in seawater-treated A549 cell, the fluorescent indicator (DCFH-DA), dihydroethidium (DHE) and MitoSox Red fluorescent probes was used to monitor generation of







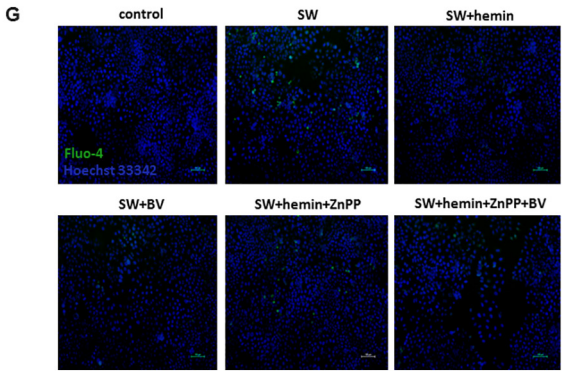


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Fig. 3. The effect of HO-1 and BV on ROS formation and maintaining mitochondrial function in A549 cell. A. Images of ROS levels by DHE fluorescent probes (10 μM). B. ROS generation was determined using DCFH-DA fluorescent probes (10 μM). C. Fluorescence images of MitoSox Red (5 μM) and Hoechst 33342 (1 μg/ml) costaining in cells. Red, cellular ROS; blue, nucleus. D and E: MMP with Rh 123 (5 μM) staining was monitored using fluorescence microscope and a microplate reader. F. Fluorescent images of JC-1 (5 μM) staining. The ratio of red and green fluorescence reflects the change of MMP. G. Fluorescent images of Fluo-4 (5 μM) staining. Green, cellular calcium flux; blue, nucleus. Values represented mean ± SD (n = 5 for each group). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. SW group; $^{\hat{}}P < 0.05$ vs. hemin + SW group; $^{\&}P < 0.05$ vs. hemin + ZnPP + SW group. Scale bars, 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intracellular ROS. All of three detection method consistently showed that seawater exposure caused a significant increase with intracellular ROS in A549 cell, which was decreased by hemin and BV treatment (Fig. 3A–C). ZnPP treatment enhanced generation of ROS in seawater + hemin + ZnPP group. We detected the mitochondria membrane potential (MMP) with the rhodamine 123 and JC-1 staining assays. The results showed that hemin and BV attenuated the mitochondrial damage (mitochondrial depolarization) while the protective effect of hemin was abrogated by ZnPP in seawater-treated cell (Fig. 3D–F). Additionally, hemin and BV decreased the intracellular calcium ion flux while ZnPP abolished this effect, which indicated by the increase in Fluo-4 green fluorescence intensity (Fig. 3G). These data suggested that HO-1 and BV protected cells by reducing ROS generation and attenuating mitochondrial damage in seawater-treated A549 cell.

3.4. The increased expression and activity of HO-1 in seawater drowning-induced lung injury in mice

The gross anatomy of lung tissue showed obvious pulmonary hyperemia and edema at 3 days after seawater drowning while these abnormalities attenuated at 7 days and 15 days after seawater drowning in mice (Fig. 4A). The lung wet/dry weight ratio and lactate dehydrogenase activity markedly increased at 3 days and 7 days after seawater drowning and decreased at 15 days after seawater drowning (Fig. 4B and D). H&E-stained sections showed that seawater exposure caused interstitial edema, hemorrhage, alveolar disarray and neutrophil infiltration at 3 days and 7 days after seawater drowning and these pathological changes ameliorated at 15 days after seawater drowning in the lung tissue (Fig. 4C). The activity of SOD significantly reduced at 3 days and 7 days and restored at 15 days after seawater drowning (Fig. 4E). Furthermore, the expression and activities of HO-1 began to significantly increase at 3 day after seawater inhalation (Fig. 4F–G). The results suggested that the enhanced expression and activity of HO-1 existed during the self-repair process after seawater drowning in mice.

3.5. Inhibition of HO-1 activity aggravated seawater drowning- induced lung injury in mice

As shown in Fig. 5A–B, 50 μmol/kg ZnPP could significantly inhibit the activities of HO-1 in seawater drowning + ZnPP group when compared with the seawater drowning group. The picture of gross anatomy in the lung tissue showed that ZnPP administration markedly aggravated pulmonary hyperemia and edema in seawater drowning + ZnPP groups when compared with that of seawater drowning group (Fig. 5C). The lung wet/dry weight ratio and lactate dehydrogenase activity were higher in seawater drowning + ZnPP groups than seawater drowning groups (Fig. 5D and F). As H&E stained results showed, seawater drowning + ZnPP groups caused more severe lung injury than seawater drowning groups, evidenced by interstitial edema, hemorrhage, alveolar disarray and neutrophil infiltration (Fig. 5E). The activities of SOD significantly reduced in seawater drowning + ZnPP groups when compared with seawater drowning groups (Fig. 5G). These data suggested that inhibition of HO-1 activity aggravated seawater drowning-induced lung injury in mice.

3.6. Inhibition of HO-1 activity impeded the self-repair ability in seawater drowning-induced lung injury in mice

Delayed self-repair would result in exuberant scar formation and fibrosis and then impaired pulmonary function [24]. To further determine the role of HO-1 in the self-repair of lung tissue after seawater drowning-induced lung injury, we first detected the levels of pulmonary fibrosis with sirius red staining and found that area with sirius red staining in seawater drowning + ZnPP groups significantly increased compared with seawater drowning groups (Fig. 6A). Nextly, the cell proliferation and apoptosis of alveolar epithelial cells were analyzed by TUNEL assay and immunohistochemistry with anti-pan Cytokeratin antibody. We noticed that pan Cytokeratin staining markedly decreased in the alveolar epithelium and the distal airway epithelium in seawater drowning + ZnPP groups compared with seawater drowning groups (Fig. 6B–C). Compared with seawater drowning groups, ZnPP administration promoted apoptosis and inhibited cell proliferation in seawater drowning + ZnPP groups. In summary, these data suggested that inhibition of HO-1 activity impaired the self-repair ability in seawater drowning-induced lung injury in mice.

4. Discussion

Seawater drowning has become the third leading cause of accidental death [25]. Approximately 50,000 people annually die mainly due to ALI or ARDS while no specific and effective treatments are currently available [26,27]. Thus, it is very important to understand the key mechanisms and look for strategies to treatment seawater-induced ALI. Many factors such as oxidation, inflammation, apoptosis and delayed cell proliferation are the key pathogenesis of seawater drowning-ALI [4]. Our previous studies demonstrated that HO-1 dramatically increased during the process of repairment in seawater drowning mice. However, the roles and mechanisms of HO-1 on seawater drowning-ALI remind to be determined. In this study, we demonstrated that seawater decreased A549 viability, increased the production of pro-inflammatory cytokines (IL-6, IL-8 and TNF-α), induced cell apoptosis and inhibited the expression of cell proliferation-related cytokines (FoxM1, Ccnb1 and Cdc25C). Moreover, seawater exposure led to mitochondrial dysfunction in A549 cell. Supplement of HO-1 sepcific inducer (heme) or its catalytic product (biliverdin) attenuated seawater-induced A549 damage and promoted cell proliferation. Mechanistically, HO-1 expression and activity persistently increased during the repair process while Zinc protoporphyrin hampered the repair process of seawater drowning-induced ARDS. This study provides many convincing evidences that HO-1 play important protective role in seawater drowning-induced lung injury (Fig. 7), which implied that HO-1 could be used therapeutically to treat drowning-induced ALI.

Anoxia and mitochondrial dysfunction are the most detrimental consequences of drowning. When suddenly immersed in water, victims hold their breath, which eventually become hypercarbic and hypoxemic [28]. In this study, we demonstrated for the first time that seawater exposure directly caused a significant increase of ROS in A549 cell; Rhodamine 123 and JC-1 staining assays further showed that seawater exposure led to mitochondrial damage. Importantly, hemin and BV attenuated the mitochondrial damage while the protective effect of hemin was abrogated by ZnPP in seawater-treated cell. In vivo study showed that ZnPP also decreased the SOD in mice after seawater

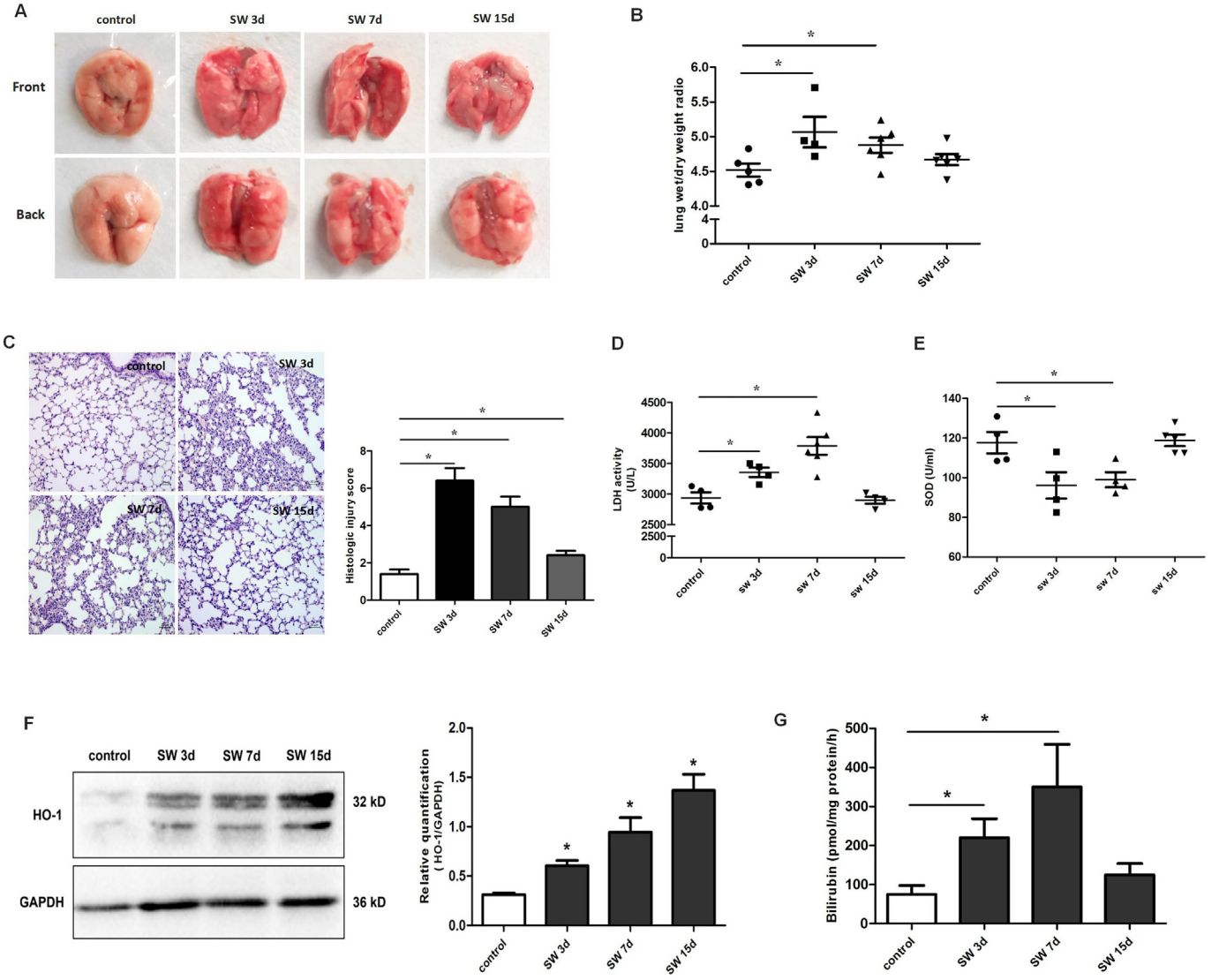

Fig. 4. The expression and activity of HO-1 increased in seawater drowning-induced lung injury in mice. A. The gross morphology of lung tissue. B. Lung wet/dry weight ratio. C. H&E stain for pathological morphology of lung tissue and lung injury scores. Scale bars, 50 μm. D. Serum LDH activity. E. SOD activity of lung tissue. F. Representative western blot images and the analysis to quantify HO-1 levels. G. HO-1 activity. Values represented mean ± SD (n = 5 for each group). *P < 0.05 vs. control group.

drowning. These results suggested that HO-1 mainly protected cells by reducing ROS generation and attenuating mitochondrial damage in seawater-treated A549 cell and mice.

Inflammation response is the prominent feature in seawater drowning-induced pulmonary injury. The hyperosmolar seawater increased infiltration of inflammation cells into the alveolar spaces and releases proinflammatory cytokines, such as TNF-α and IL-6 [29,30]. Folkesson et al. [31] indicated that seawater instilling into the trachea of rabbits increase alveolar-capillary membrane permeability, resulting in the exudation of water, ions and proteins, as well as neutrophils and macrophages. Seawater induced the release of inflammatory cytokine through multiple pathways, including nuclear factor-κB [30], hypoxia-inducible factor-1α [32], macrophage migration inhibitory factor [33] and RhoA/Rho kinase signaling [34]. In addition, neutrophilic activation also promotes the release of reactive oxygen species (ROS). Consequently, massive inflammatory mediators are produced and elicit serious damage to lung tissue and cells. In support with this notion, this study firstly revealed that seawater could enhance the production of TNF-α, IL-6 and IL-8 in A549 cell. Nextly, we investigated the effect of HO-1 on the production of pro-inflammatory cytokines and found that hemin decreased the content of TNF-α, IL-6 and IL-8 in seawater-treated A549 cell; ZnPP elevated these pro-inflammatory cytokines; BV could abrogate the effect of ZnPP in the production of pro-inflammatory cytokines. In seawater-drowning mice, H&E-stained sections showed that seawater exposure caused neutrophil infiltration and HO-1 activity inhibition aggravated seawater drowning-induced ALI in mice. However, the detailed molecular mechanisms about seawater drowning-induced HO-1 up-regulation remains unknown. Although it has been widely accepted that Keap1/Nrf2 system plays a central role for HO-1 induction in response to oxidative stress, other transcription factors such as NF-κB and AP-1 also can increase HO-1 content [35]. Moreover, cellular signaling transducers including p38 MAPK and phosphatidylinositol-3 kinase/Akt are responsible for HO-1 regulation [36]. Due to the complexity of the regulatory mechanism of HO-1 expression, we will employ conditional Nfe2l2/Hmox1 double knockout mice to explore the role of Keap1/Nrf2 system on HO-1 expression in seawater drowning-induced ALI.

Seawater is a hyperosmotic liquid which contains a high content of sodium, calcium and substantial quantities of bacteria. Seawater is three times more hyperosmolar than plasma (942 vs. 300 mOsm/kg,

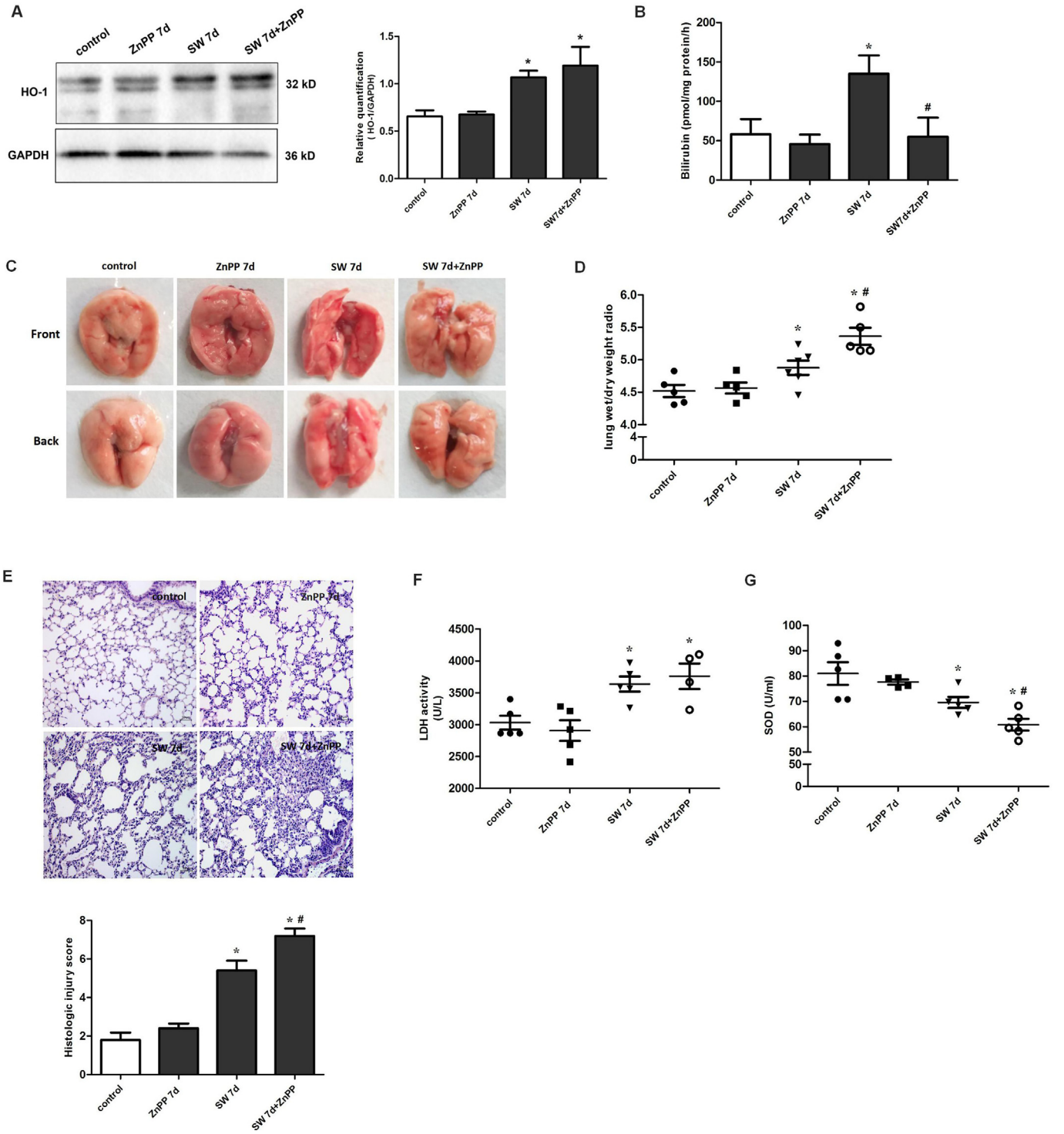

Fig. 5. Inhibition of HO-1 activity by ZnPP administration aggravated seawater drowning-induced lung injury in mice. A. Representative western blot images and the analysis to quantify HO-1 levels. B. HO-1 activity. C. The gross morphology of lung tissue. D. Lung wet/dry weight ratio. E. H&E stain for pathological morphology of lung tissue and lung injury scores. Scale bars, 50 μm.
F. Serum LDH activities. G. SOD activities in lung tissue. Values represented mean ± SD (n = 5 for each group). *P < 0.05 vs. control group; #P < 0.05 vs. SW 7d group.

respectively). The severity of seawater-induced pulmonary edema is 3-fold higher than that caused by freshwater [37]. Hypertonic fluids stimulation cause many pathological perturbations in lung tissue, such as lung epithelial and vascular endothelial cell apoptosis, shrinkage, blood-gas barrier damage, as well as infiltration and secretion of inflammatory cytokines [31,38]. HO-1 activity had pro-proliferative effects in vascular endothelial cells [39]; HO-1 knockdown suppressed proliferation in endothelial cells [40]. In this study, we firstly found that seawater destroyed the cell integrity and viability in time-dependent manner; the result of flow cytometry revealed that seawater induced A549 cells apoptosis. We also measured the expression changes of cell proliferation-related cytokines (FoxM1, Ccnb1 and Cdc25C) in

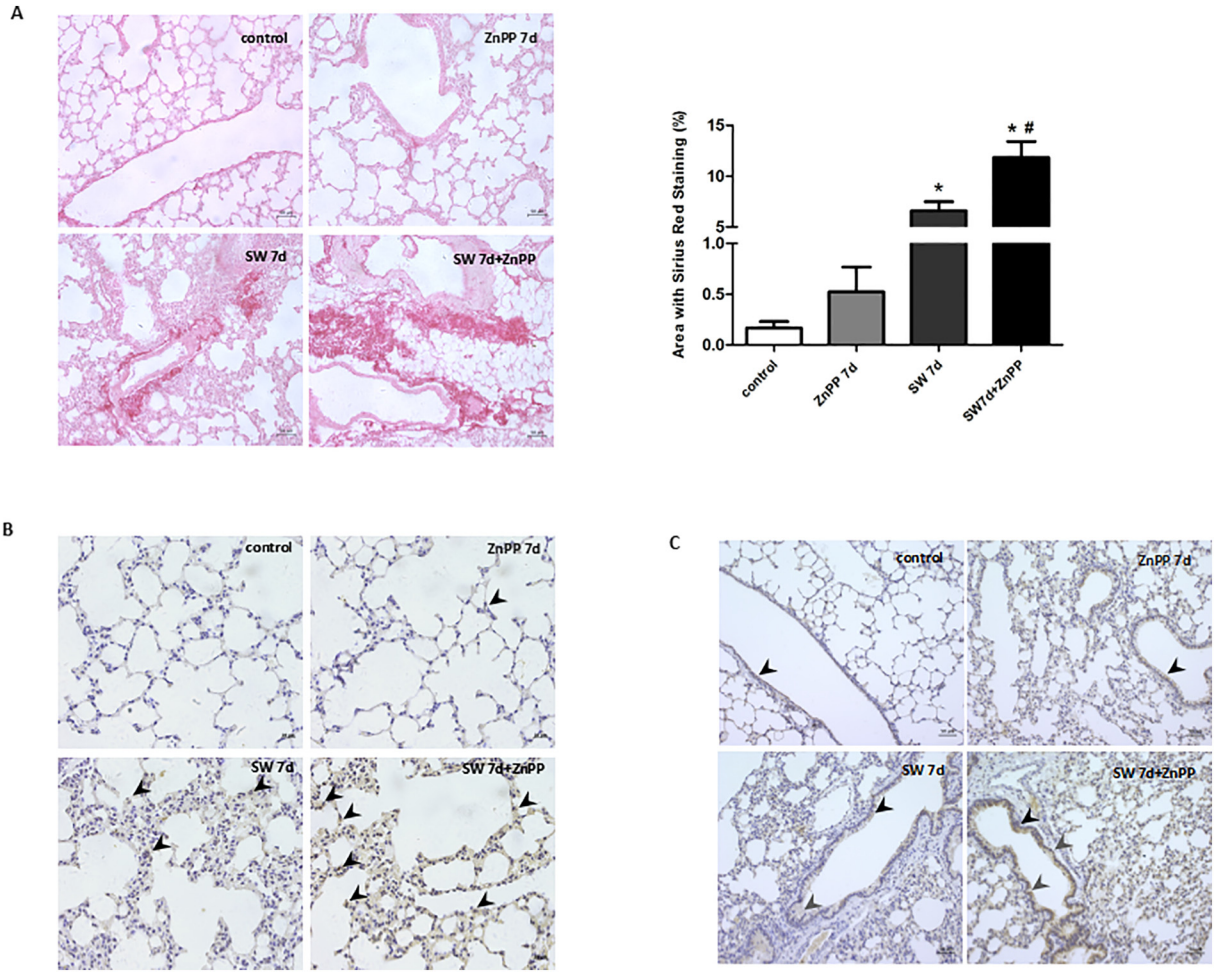

Fig. 6. Inhibition of HO-1 activity by ZnPP administration impeded the self-repair in seawater drowning-induced lung injury in mice. A. Representative images of Sirius red staining and statistical analysis of images in Sirius Red staining. B Representative images of TUNEL assay. Black arrow heads: positive TUNEL assay. C Immunohistochemistry for pan Cytokeratin. Black arrow heads: epithelial cells; gray arrowheads: defect in epithelium. Values represented mean ± SD (n = 5 for each group). $^{*}P < 0.05$ vs. control group; $^{\#}P < 0.05$ vs. SW 7d group. Scale bars, 50 μm.

seawater-treated A549 cell. The cell proliferation assays showed seawater could induce cell apoptosis and inhibit the expression of cell proliferation-related cytokines in A549 cell. Hemin and BV could inhibit cell apoptosis and increase the expression of cell proliferation-related cytokines (FoxM1 and Ccnb1) in seawater-treated A549 cell. The effect of hemin was abrogated by ZnPP while BV treatment could overcome the adverse effect of ZnPP. We further analyzed the proliferation and apoptosis of alveolar epithelial cells by TUNEL assay and staining with anti-pan Cytokeratin antibody in mice. Pan Cytokeratin staining markedly attenuated in the alveolar epithelium and the distal airway epithelium after ZnPP treatment; ZnPP administration promoted apoptosis and inhibited cell proliferation. In summary, these data suggested that inhibition of HO-1 activities impeded the self-repair in seawater drowning-induced lung injury in mice.

This study supported the notion that HO-1 plays an important role in attenuating seawater drowning-induced lung injury, HO-1 and BV have been reported to have cytoprotective effect through its anti-oxidative and anti-inflammatory function on many pulmonary diseases [41,42]. Nevertheless, microsatellite $(GT)_n$ dinucleotide length polymorphisms in the regulatory regions of the human *HMOX1* gene promoter affect HO-1 promoter transcriptional activity. The number of GT repeats was negatively correlated with *HMOX1* transcriptional activity [43]. Individuals with the long (L) allele $[(GT)_n \geq 30]$ have lower content of HO-1 than the short (S) allele $[(GT)_n < 25]$. Patients with longer *HMOX1* promoters suffered from more severe injury cause by stress-associated diseases such as myocardial infarction than patients with shorter *HMOX1* promoters probably due to the lower HO-1 levels [44]. As for the patients with the longer *HMOX1* promoters, exogenous HO-1 byproduct supplement maybe is an important means to treat HO-1 associated diseases. Luckily, supplementation of HO-1 bioactive products (CO and BV) has been found to dampen the inflammatory response and prevent from tissue injury [45,46]. This study also show that HO-derived byproducts, BV, exhibit similar protective effects on seawater drowning-induced lung injury in mice. Therefore, BV may act as a new compound to treat seawater drowning-induced lung injury especially for drowning victims with the long (L) allele [(GT) n ≥ 30] in HO-1promoter.

5. Conclusion

HO-1 attenuates seawater drowning-induced lung injury by its anti-oxidative, anti-inflammatory, and anti-apoptosis properties.

Abbreviations

HO-1	heme oxygenase-1
ALI	acute lung injury
ARDS	acute respiratory distress syndrome
IL-6	interleukin-6
IL-8	interleukin-8

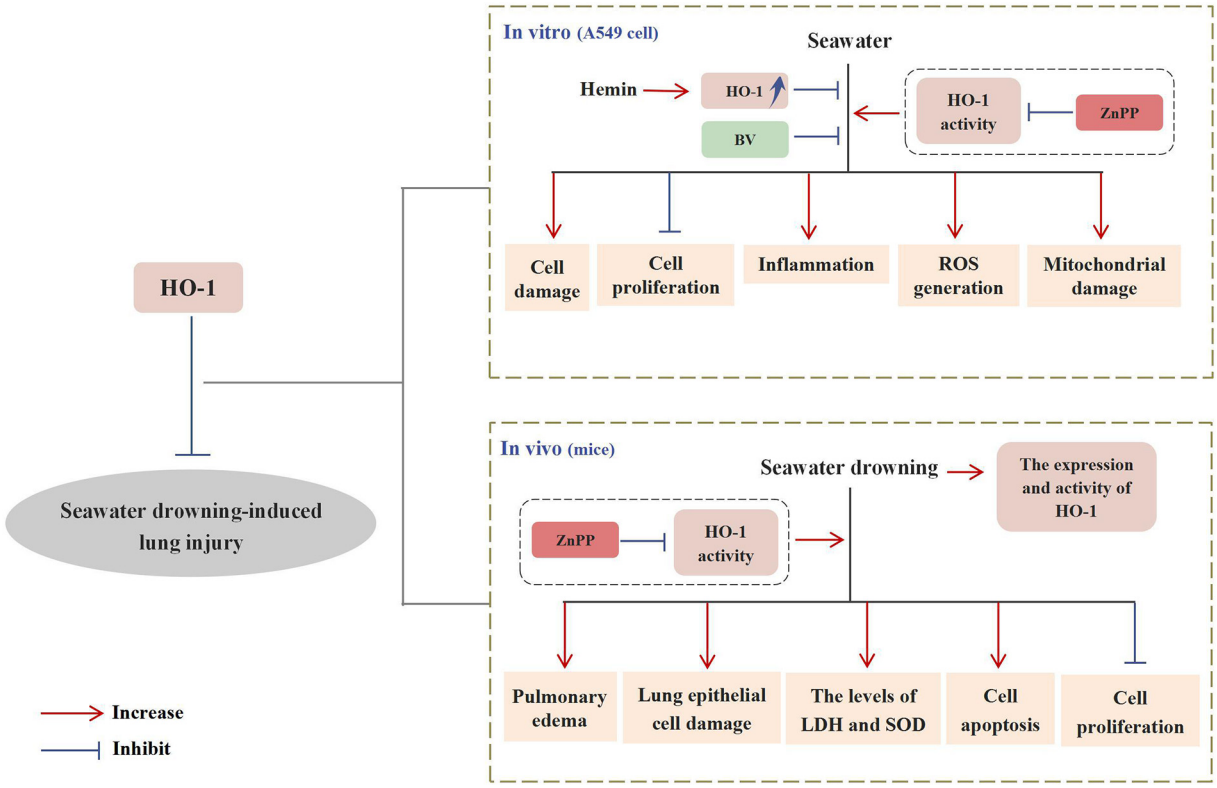

Fig. 7. The proposed protective mechanism of HO-1 on seawater drowning-induced lung injury.

TNF-α	tumor necrosis factor-α
FoxM1	Forkhead Box M1
Ccnb1	G2/mitotic-specific cyclin-B1
Cdc25C	cell division cyclin 25 homolog C
MCP-1	monocyte chemoattractant protein-1
SW	seawater
BV	biliverdin
ZnPP	zinc protoporphyrin IX

Declaration of Competing Interest

The authors have no conflicts of interest to report.

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