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ORIGINAL ARTICLE

Benefit of Broccoli Extract-Sulforaphane Prophylaxis in Ventilator-Induced Lung Injury

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Background: Owig to the extensive use of mechanical ventilation, risks of acute lung injury are significant in the intensive care unit. Broccoli extract-sulforaphane (SFN) has been investigated as bioactive polyphenol in chronic lung diseases. **Aim:** The present study aimed to evaluate the preventive effect of SFN in a rat model of ventilator-induced lung injury. **Methods:** SFN supplement was administrated 30 min before intubation with the dosage of 3 mg/kg. Then, rats were assigned to receive ventilation with a high tidal volume of 40 mL/kg for 6 h, and low ventilation of 6 mL/kg served as controls. **Results:** The severity of pulmonary edema was mitigated in the SFN-pretreated group with decreased weight ratios of wet to dry lung and total lung to the body, respectively. From bronchoalveolar lavage, SFN treatment suppressed both leukocytes counts and cytokines production. Following ventilator-exerted oxidative burst with the rescue of glutathione level was identified in SFN-pretreated group. Besides, SFN-reduced cell apoptosis was confirmed by terminal deoxynucleotidyl transferase dUTP nick end labeling assay and cleavage of caspase-3. Western blotting from lung tissues revealed the upregulation of hemeoxygenase-1 with decreased nuclear factor κB and p38 phosphorylation in SFN-treated group. **Conclusion:** Our results elucidated the prophylaxis of broccoli extract-SFN could attenuate ventilator-induced oxidative stress, inflammation reaction, and pulmonary edema.

Key words: Sulforaphane, polyphenol, ventilator-induced lung injury, nuclear factor-kappa B, heme oxygenase-1, oxidative stress

INTRODUCTION

Micronutrient supplementation comprising vitamins and trace elements has rapidly gained interest in the critical care field, particularly in alleviating oxidative stress.[1] In ventilated patients through enteral/parenteral feeding solutions, micronutrients such as polyphenols are frequently unavailable than generally acknowledged.[2-4] Broccoli is recognized as a super-food due to the high content of polyphenols and sulforaphane (SFN), a major bioactive compound with excellent anti-inflammation properties.[5-7] In the field of chronic lung diseases, few publications had discussed the benefit of SFN in asthma and chronic obstructive pulmonary disease.[8] Furthermore, SFN pretreatment suppressed leukocyte infiltration in human subjects exposed to diesel exhaust particles.[9] The therapeutic efficacy of SFN had been explored in several models of lung injury. The administration of SFN was found to alleviate inflammation and exert oxidative stress in mice of oxidant-induced lung injury, rabbits with acute respiratory distress syndrome, and lipopolysaccharide (LPS)-induced acute lung injury (ALI) through nuclear factor erythroid 2 (Nrf-2) signaling.[10-12] However, the potential of SFN as pharmaco-nutrition has not been explored in ALI induced by mechanical ventilation.

Acute respiratory failure is one of the most common causes for intensive care unit admission. In contrast, indispensable mechanical ventilation may damage lung parenchyma through physical stress, so-termed "ventilator-induced lung injury" (VILI).[13] In the early 1970s, scientists have observed

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the development of perivascular and alveolar edema within 35 min in ventilated rats.[14] Other physiological abnormalities included elevated wet-to-dry ratio of the isolated lung, composite alterations in bronchoalveolar lavage fluid (BALF), and increased thickness of hyaline membranes.[15] At the cellular level, VILI is characterized by the release of cytokines, including interleukin-1 (IL-1) β and tumor necrosis factor-α (TNF-α), recruitment of leukocytes, predominately neutrophils, and massive apoptosis of pneumocytes.[16,17] Clinical practice has mainly focused on ventilation strategies, whereas pharmacological interventions remain uncertain areas, especially inflammation-targeted agents.[18] Taken advantage of high bioavailability and less systemic toxicity, natural extracts have emerged as a promising drug class in ALI.

Despite better understanding of pathophysiology in ALI, clinicians still lack consensus for its systemic pharmacotherapy. These experimental therapies encompass prostaglandin E1 (PGE1) as a vasodilator, glucocorticoid as an anti-inflammatory agent, and N-acetylcysteine (NAC) as an anti-oxidant drug.[19] PGE1 has the potency to modulate neutrophils activation, whereas its nonselective vasodilatory effects exert severe hypotension.[20,21] Although steroids have been experimented in the prevention and treatment of acute respiratory distress syndrome,[22] its inhibition of host defense is highly concerned. As NAC alleviates oxidative stress, the evidence to support its clinical use is not adequate.[23] Therefore, the development of the new drug in ALI is in urgent need. The present study aimed to assess the prevention use of broccoli concentrate-SFN in the rat VILI model through mitigating leukocyte infiltration, oxidative burst, and lung edema.

MATERIALS AND METHODS

Animals

Taipei Veterans General Hospital Research Animal Care Subcommittee approved the study (IACUC-2012-201). Sprague − Dawley virus-free rats weighing between 250 and 300 g were obtained from National Laboratory Animal Center, Taiwan.

Experimental protocols

Male Sprague − Dawley rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20–25 mg) while breathing room air. PE 240 tubing was inserted into the trachea and connected to a Harvard apparatus ventilator, model 55-7058 (Holliston, MA, USA). We used our established ventilator protocol in the rat model of VILI as previously described.[24] The rats were then randomly assigned into four groups ($N = 7$ for each group) and ventilated for 6 h. These groups consisted of: (a) a control group using a tidal volume (VT) of 6 ml/kg, (b) a vehicle control group using dimethyl sulfoxide (DMSO), (c) a VILI group using a high VT of 40 ml/kg, and (d) a VILI + SFN treatment group. R, S-SFN, purchased from LKT Laboratories, Inc. (Minnesota, USA) was prepared with DMSO and given 3 mg/kg 30 min before starting the mechanical ventilator. A positive end-expiratory pressure of 2 cm H2O was applied in all groups. End-tidal CO2 was monitored intermittently by a microcapnograph (Columbus Instruments, Columbus, OH, USA) and was kept between 35 and 45 torrs by adjusting the respiratory rate of the ventilator. The femoral artery and vein were cannulated. Tracheal airway pressure and arterial blood pressure were monitored with a polygraph (Gould Instruments, Cleveland, OH, USA). During the period of ventilator use, intra-peritoneal sodium pentobarbital (0.05 mg/g) was administered every 30 min, and the intraperitoneally administered anesthetic fluid was sufficient to correct for hypovolemia. The chest was opened by incision of the left border of the sternum 6 h after the experiment.

Bronchoalveolar lavage fluid

The lungs were removed *en bloc*, and tubing was inserted into the trachea and secured. The right lung was clamped at the bronchus to prevent lavage fluid from entering. The left lung was lavaged by instilling 2.5 mL of normal saline twice. The recovered lavage samples were centrifuged at 1,500 g at the room temperature (RT) for 10 min. The supernatant was stored in a –80 ℃ refrigerator for the later measurement of cytokines. The white cell count in the BALF was determined by using a hemocytometer. Measurement of the concentration of cytokines, including macrophage inflammatory protein 2 (MIP-2), IL-1 β, and TNF-α, was performed by enzyme-linked immunosorbent assay kits (RandD Systems, Oxon, UK).

Measurement of hydrogen peroxide

BALF was centrifuged at 1,000 g within 30 min, the supernatant was collected, and 50 μl hydrogen peroxide (H_2O_2) reaction mix containing 46 μl assay buffer, two μl OxiRed™ probe solution, and 2 μl HRP solution (BioVision, USA) was added to the supernatant of the BALF and incubated for 10 min. Absorbance was read at 570 nm (SpectraMax M5; Molecular Devices, USA). The concentration was calculated based on H_2O_2 standard curves.

Measurement of thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation diagnostic for tissue damage produced by oxidative stress. The TBARS level in the BALF supernatant was measured using the OxiSelectTM TBARS assay kit (Geneteks Biosciences, Inc.).

Measurement of myeloperoxidase, protein carbonyl, and reduced glutathione levels in lung tissue

The concentration of myeloperoxidase (MPO) in the right middle lung tissue, an index of neutrophil sequestration in the lungs, was measured as previously described. Lung tissue protein carbonyl content was measured by protein carbonyl assays (Geneteks Biosciences, Inc., San-Chong City, Taipei). Measurement of glutathione concentrations was carried out according to the method described by a commercially available kit (Sigma–Aldrich Fine Chemicals, Munich, Germany).

Western blotting analysis

Lung tissues were homogenized using lysis buffer containing a protease inhibitor cocktail (Roche, USA) and a phosphatase inhibitor cocktail (Roche, USA). Total protein extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred onto polyvinylidine fluoride membrane (Millipore, USA). The membrane was blocked with 5% nonfat dry milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 h. Antibodies against phospho-p44/42 mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase [ERK] 1/2), phospho-stress-activated protein kinases/Janus kinase/signal transducers and activators of transcription (JNK), and phospho-p38 MAPK (1:1,000; Cell Signaling Technology, Beverly, MA, USA) were used. Antibodies against glyceraldehyde 3-phosphate dehydrogenase (GADPH, 1:10,000; Lab Frontier, Korea), JNK (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3 (1:2,000; Cell Signaling Technology), p-AKT (1:1,000; Cell Signaling Technology), AKT (1:1,000; Cell Signaling Technology), and heme oxygenase-1 (HO-1, 1:1000, Abcam, UK), were also used. The appropriate secondary antibodies were used (1:10,000 horseradish peroxidase anti-rabbit; Jackson Immuno Research Laboratories, West Grove, PA, USA). Visualization was performed by enhanced chemiluminescence (Visual Protein Biotechnology Corp., Taiwan). Protein bands were quantified using Kodak 1D image analysis (Eastman Kodak Company, Rochester, NY, USA).

Nuclear factor κB analysis of nuclear protein

Lung tissue was homogenized with a Dounce tissue homogenizer in 5-mL of Solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged for 30 s at 2,000 rpm, and the supernatants were collected and centrifuged for 5 min at 5,000 rpm. The pelleted nuclei were resuspended at 4°C in 300 μL of solution B (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 μg/mL pepstatin A, 5 μg/mL leupeptin, 5 μg/mL aprotinin) and incubated on ice for 20 min. Samples were centrifuged at 15,000 rpm for 1 min. The total protein concentration in the extract was determined with a bicinchoninic acid protein assay (Pierce). The membrane was blocked for 1 h. Anti-nuclear factor κB (NF-κB) antibody (1:1,000; Cell Signaling Technology) and anti-PCNA antibody (1:1,000; Cell Signaling Technology) were diluted in TBST buffer (TBS/0.1% Tween 20) and incubated at 4°C overnight. The appropriate secondary antibody was used (1:10,000 horseradish peroxidase anti-rabbit Jackson ImmunoResearch Laboratories) at RT for 1 h. Visualization was performed by enhanced chemiluminescence (Visual Protein Biotechnology Corp). The protein bands on the destained gels were quantified with the Kodak 1D Image Analysis version 3.5 software package (Eastman Kodak Company). Anti-PCNA antibody was used as a loading control to correct for the pixel values of NF-κB.

Terminal deoxynucleotidyl transferase dUTP nick end labeling stain for apoptosis

Lung slides coated with poly-L-lysine (Sigma, St. Louis, MO, USA) were deparaffinized and rehydrated using xylene and ethanol. The background was diminished by preincubating samples with 3% bovine serum albumin (BSA) and 20% normal bovine serum in PBS for 30 min at RT. The specimens were then exposed for 1 h at 37°C in a moist chamber to a labeling mix containing 0.135 U/mL calf TdT, 0.0044 nmol/mL digoxigenin-11-2'-deoxy-uridine-5'-triphosphate and 1 mM Co chloride in distilled water. Following washing, the specimens were re-saturated in 3% BSA and 20% normal sheep serum, then treated (1 h at RT) with a 1.25 peroxidase U/mL dilution of peroxidase-labeled anti-digoxigenin sheep Fab fragment, followed by washing with 0.05% 3-3'-diaminobenzidine tetrahydrochloride (Dako, USA) color reaction. Analysis was performed under an Eclipse 80i microscope (Nikon, Japan) using Image Pro Plus 5.0 (Media Cybernetics, USA). The cells with positive terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining in nuclei were counted in groups of 100 cells on 3 slides of immunohistochemical stain for each animal tissue. Two pathologists blinded to the experimental condition carried out the morphological assessments.

Statistical analysis

Systat10.0 (Systat Software Inc., San Jose, CA, USA) was used for the statistical analyses. The comparisons among all groups were conducted using an ANOVA for repeated measurements. The comparisons between baseline and post-VILI values within each group were conducted using the Student's paired *t*-test. The values are expressed

as the means ± standard deviation. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of sulforaphane on systolic pressure and heart rate

Rats were randomly assigned as (a) control group ventilated with VT of 6 ml/kg, (b) 0.1% DMSO (vehicle control) group ventilated with VT of 6 ml/kg, (c) VILI group ventilated with high VT of 40 ml/kg, and (d) SFN-pretreated group ventilated with high VT of 40 ml/kg. SFN was prepared with 0.1% DMSO and administrated 30 min before intubation with the dosing of 3 mg/kg. After mechanical ventilation for 6 h, systolic pressure and heart rate were not statistically different by SFN treatment [Table 1]. The therapeutic mechanism of SFN might not be through the regulation of hemodynamic.

Table 1: Hemodynamic effects of sulforaphane

Groups	*n*	Systolic pressure (mmHg)		Heart rate (beat/min)	
		Baseline	6 h	Baseline	6 h
Control	7	107.8±16.5	103.0±27.6	335.2±20.9	253.5±17.9
Control + DMSO	7	127.0±6.1	121.4±12.9	349.7±29.6	264.7±16.6
VILI	7	139.9±27.6	131.0±17.2	379.1±40.3	262.8±35.7
VILI + SFN	7	125.7±24.6	124.9±13.4	370.9±34.4	278.7±35.1

Oral SFN was given 30 minutes before mechanical ventilation. VILI was created by a high tidal volume setting of 40 mL/kg, and the control group underwent a low tidal volume setting of 6 mL/kg. Systolic blood pressure and heart rate were recorded at the indicated time point via a femoral artery catheter. Value presented as mean±SD. VILI=Ventilator-induced lung injury; SD=Standard deviaton; SFN=Sulforaphane; DMSO=Dimethyl sulfoxide

Decreased severity of lung edema and inflammation in sulforaphane -pretreated group

The lung wet to dry (W/D) ratio served as an indicator of pulmonary edema, as well as a total lung to body weight ratio. Following 6 h ventilation, the VILI group showed a significant increase in both ratios of lung W/D and total lung to body weight [Table 2]. Compared with the VILI group, SFN pretreatment significantly suppressed the severity of pulmonary edema. The number of white blood cells and cytokines level in BALF reflected pulmonary inflammatory status. High VT ventilation recruited leukocytes infiltration in VILI [Table 2]. However, fewer leukocytes count was identified in the SFN-pretreated group. The lavage fluid analysis revealed a general increase in cytokine levels and oxidative stress after 6 h of high VT ventilation [Figure 1a]. Furthermore, SFN suppressed VILI-induced proinflammatory cytokines and oxidative stress, such as TNF-α, IL-1 β, MIP-2, H_2O_2, and lipid peroxidation (TBARS). MPO activity has a positive correlation with neutrophils infiltration. Compared with the VILI group, MPO activities were lower in SFN pretreated group [Figure 1b]. Besides, pretreated SFN diminished VILI-induced protein decarbonylation and restored the glutathione level. In conclusion, rats with oral SFN supplement showed a protective effect against ventilator-induced pulmonary edema, inflammation, and oxidative stress.

Table 2: Effects of sulforaphane on lung edema and bronchoalveolar lavage fluid analysis

Groups	*n*	W/D	Cell count (μl)	L/B
Control	7	0.261±0.014	68.8±13.4	0.0019±0.0004
Control + DMSO	7	0.250±0.017	85.9±13.8	0.0037±0.0003
VILI	7	0.293±0.051$^{\#}$	201.0±21.7$^{\#}$	0.0074±0.0014$^{\#}$
VILI + SFN	7	0.266±0.023$^{\&}$	127.3±21.4$^{\#,\&}$	0.0044±0.0011$^{\#,\&}$

After 6 h mechanical ventilation, rats were sacrificed to calculate ratios of lung wet to dry and lung to body weight. The left lung was lavaged by 2.5 ml normal saline twice to collect BALF, and white blood cell counts were measured by hemocytometer. $^{\#}$Indicates a significant difference with the control group ($P<0.05$); $^{\&}$Indicates a significant difference with the VILI group ($P<0.05$). The value presented as mean±SD. SD=Standard deviaton; SFN=Sulforaphane; DMSO=Dimethyl sulfoxide; VILI=Ventilator-induced lung injury; BALF=Bronchoalveolar lavage fluid

Effect of sulforaphane against apoptosis and nuclear factor κB activation

Ventilation has been observed to trigger cell apoptosis. TUNEL assay was applied to detect apoptosis activity in lung tissue sections. Increased immunostaining was noticed in the VILI group with massive alveolar collapse [Figure 2a]. In comparison, the SFN-pretreated group revealed decreased apoptosis with relatively intact alveolar space. Western blotting showed consistent results that cleavage caspase-3 expression was suppressed under SFN treatment [Figure 2b]. The pathogenic role of Nf-κB has been confirmed in ALI. Following 6 h ventilation, there was an upward trend of Nf-κB phosphorylation [Figure 2b]. Furthermore, SFN treatment could reverse ventilator-induced Nf-κB nuclear translocation. As a result, SFN pretreatment had the potency to inhibit apoptosis signaling and Nf-κB activation in the VILI model.

Blockade of p38 phosphorylation and elevated hemeoxygenase-1 expression by sulforaphane pretreatment

The continuous physical force under ventilation was associated with marked activation of MAPK and AKT signaling. There was elevated phosphorylation of ERK, JNK, and p38 but not AKT in lung tissue homogenates [Figure 3a-c and 3e]. Among the above signaling, SFN pretreatment exclusively mitigated p38 activation in the VILI group. Upraised HO-1 expression was beneficial in ALI through

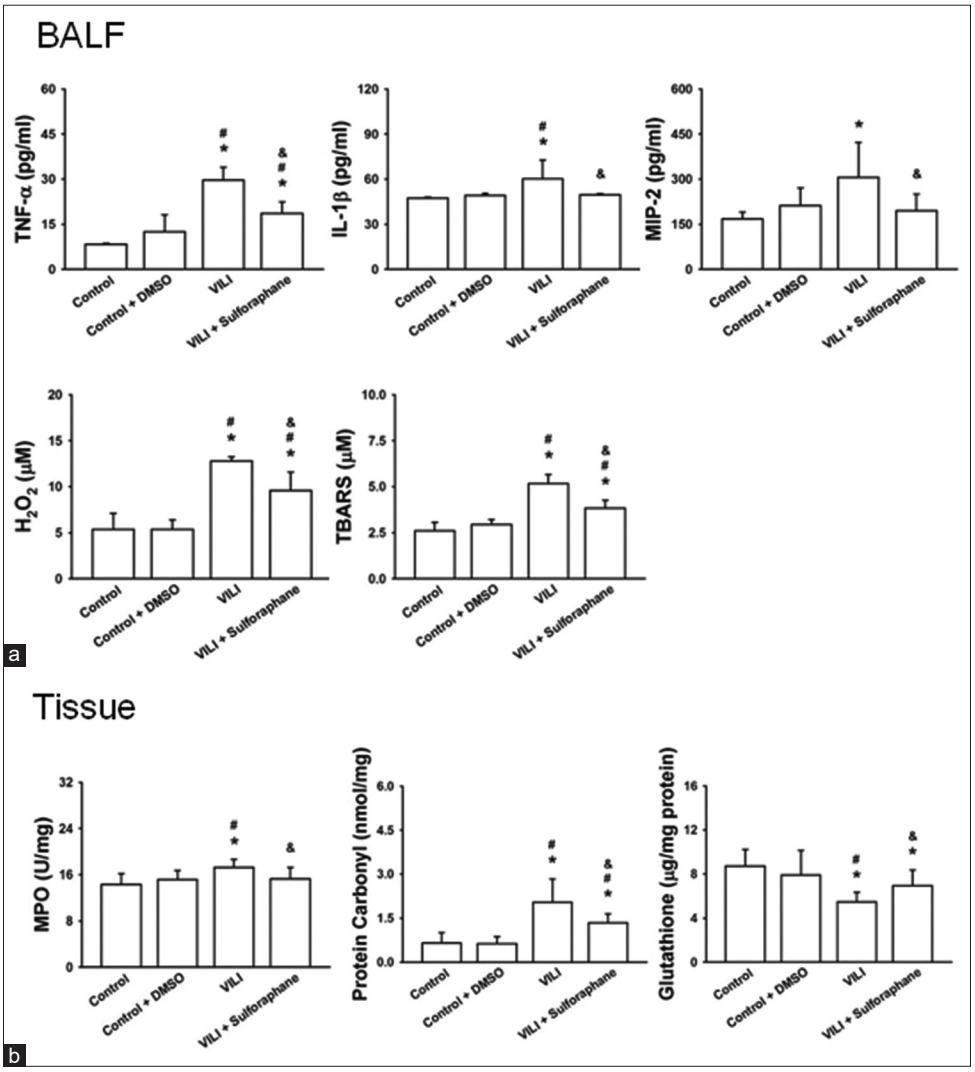

Figure 1: Sulforaphane diminished cytokines level, oxidative stress, and neutrophils recruitment in bronchoalveolar lavage fluid and lung tissue homogenates. (a) Using enzyme-linked immunosorbent assay kits, bronchoalveolar lavage fluid samples were examined with concentrations of tumor necrosis factor-α, interleukin-1 β, macrophage inflammatory protein 2, H_2O_2, and thiobarbituric acid reactive substances. (b) Levels of myeloperoxidase, protein carbonylation, and glutathione from the right middle lung tissue were determined by the enzyme-linked immunosorbent assay. *indicates the significant difference with the control group ($P < 0.05$), # indicates the significant difference with the control + dimethyl sulfoxide group ($P < 0.05$), indicates the significant difference with the ventilator-induced lung injury group ($P < 0.05$)

the anti-oxidant mechanism. Compared with the VILI group, SFN treatment further enhanced the HO-1 protein level under high VT ventilation [Figure 3d]. Conclusively, the therapeutic mechanism of SFN was postulated through inhibiting p38 phosphorylation with protective HO-1 expression.

DISCUSSION

Following the trend of nutraceuticals as anti-inflammation agents, the purpose of the current study is to evaluate the efficacy of broccoli extract-SFN against VILI. Rats receiving high VT ventilation resulted in ALI manifested by lung edema, cytokines release, leukocytes infiltration, and pneumocytes apoptosis. In contrast, SFN pretreatment ameliorated the above pathogenic indices with significantly decreased oxidative stress. Our *in vivo* data supported the notion that SFN is capable of alleviating VILI in a prophylactic manner.

Neutrophil recruitment and activation are regarded as the vital feature in the early phase of VILI.[25] In the present experiment, leukocytes migrated into the lungs in response to high VT ventilation, as manifested by elevation in BALF cell count and tissue MPO activity. Pretreatment with SFN remarkably attenuated inflammatory cells sequestration, as well as cytokines, MIP-2 and Nf-κB elevation. These findings

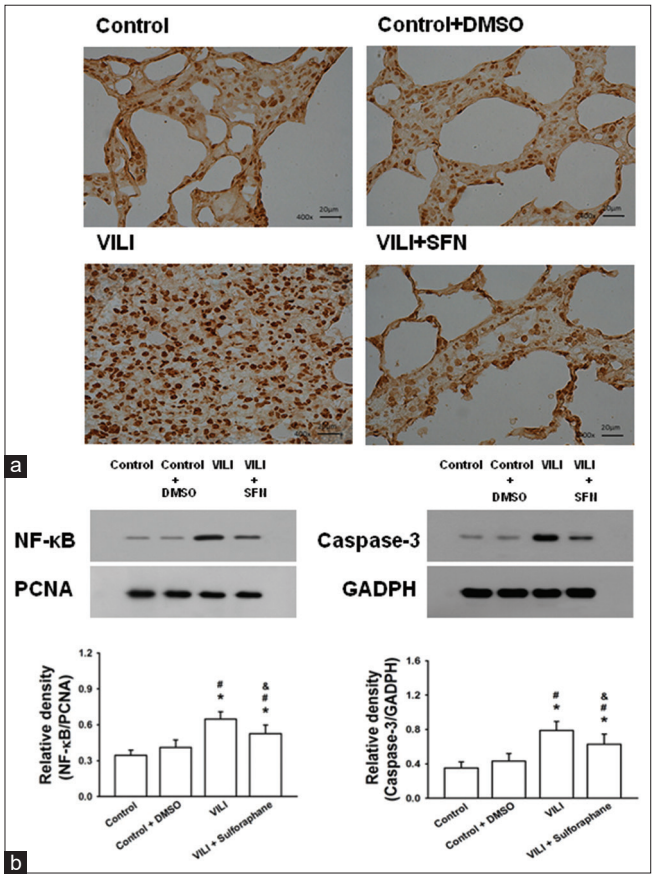

Figure 2: Sulforaphane prevented apoptosis and down-regulated nuclear factor κB activation. (a) Representative images of lung sections by the terminal deoxynucleotidyl transferase dUTP nick end labeling apoptosis assay following ventilation for 6 h. (b) Western blot analysis of nuclear factor κB and cleavage caspase-3 in lung tissues homogenates. Proliferating cell nuclear antigen and GAPDH were used as the internal control. Data were analyzed with the paired *t*-test. *indicates the significant difference with the control group ($P < 0.05$), #indicates the significant difference with the control + dimethyl sulfoxide group ($P < 0.05$), &indicates the significant difference with the ventilator-induced lung injury group ($P < 0.05$)

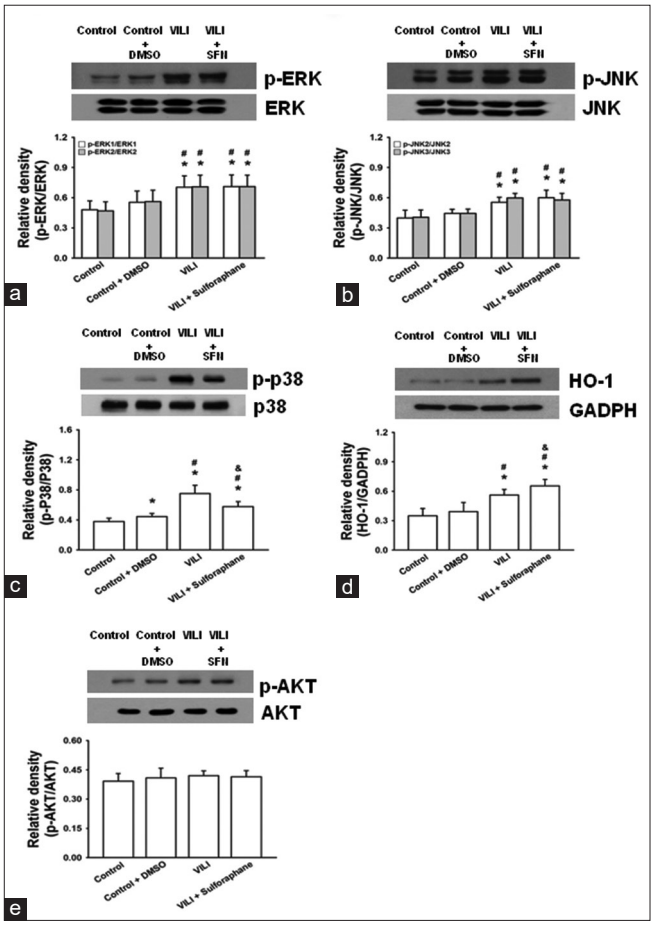

Figure 3: Effect of sulforaphane on mitogen-activated protein kinases and AKT activation, hemeoxygenase-1 expression. Western blot analysis of (a) p-Extracellular signal-regulated kinase, (b) p-Janus kinase/signal transducers and activators of transcription, (c) p-p38, (d) hemeoxygenase-1, and (e) p-AKT in lung tissues homogenates. Data were analyzed with the paired *t*-test. *indicates the significant difference with the control group ($P < 0.05$), #indicates the significant difference with the control + dimethyl sulfoxide group ($P < 0.05$), &indicates the significant difference with the ventilator-induced lung injury group ($P < 0.05$)

suggest that the protective effect of SFN could be attributed to immune-modulator against high ventilation-induced inflammation.

Apart from neutrophils accumulation, elevated oxidative stress is another feature of VILI. SFN was well-known as potent antioxidant through activation of Nrf-2 and its down-stream target HO-1.[26] Literature reviews have established SFN owned advantage of less systemic toxicity and high bioavailability.[27,28] Our results are the first to describe the treatment of VILI with SFN, which could suppress reactive oxygen species production and subsequent pneumocytes apoptosis. Moreover, there has been reported that HO-1 upregulation exerted anti-inflammatory and anti-oxidant effects in ALI.[29] Increased HO-1 expression was found under VILI conditions and further augmented in SFN-treated group. Our results suggested that SFN has a role in adjuvant therapy of VILI targeted oxidative damage with HO-1 preconditioning.

MAPK pathways have been identified as critical regulators to translate mechanical force to gene regulation in VILI development.[30] In addition, MAPKs regulated the phosphorylation of Nrf2 and ARE-mediated HO-1 gene expression.[31] Western blotting analysis showed widespread elevation of phosphorylated MAPKs in high V_T, whereas p38 MAPK signaling was exclusively mitigated under SFN pretreatment. The p38 MAPK kinase has been implicated in the mechanotransduction-associated apoptosis.[32] Moreover, increased p-p38 was correlated with MIP-2 production in the study of LPS -induced lung injury.[33] Our results elucidated therapeutic mechanism of SFN treatment through reduced p38 activation and inflammatory cytokine cascades.

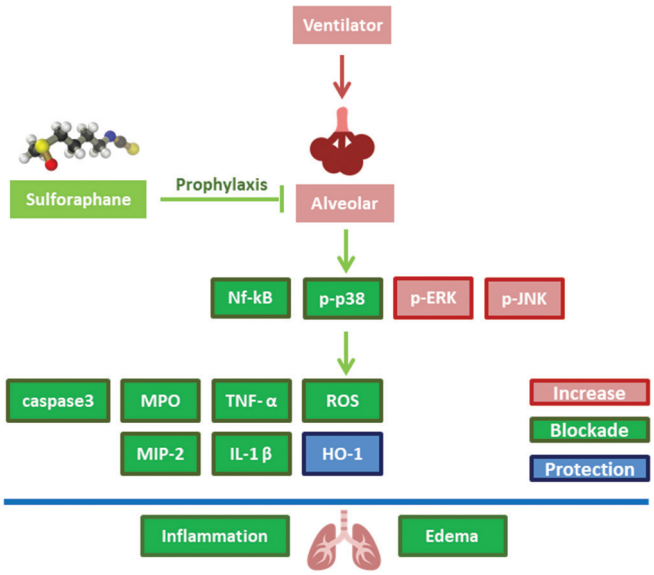

Figure 4: Schematic presentation for sulforaphane in ameliorating ventilator-induced lung injury. Mechanical ventilation induces lung injury through inflammatory cytokines and neutrophils recruitment. Sulforaphane upregulates heme oxygenase-1 and subsequently eases the inflammation by reducing P38 and nuclear factor κB activation to minimize ventilator-induced lung inflammation and edema

CONCLUSION

The application of SFN can effectively attenuate VILI through the inhibition of inflammation and reduction of oxidative stress and apoptosis in a high V_T-induced lung injury rat model [Figure 4]. Our results hinted that bioactive food compound-SFN might function mechanistically to elevate HO-1, and subsequently proceed through the reduction of P38 and NF-κB activation to exert its protective effects.

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Conflicts of interest

There are no conflicts of interest.

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