Research Square

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Seabuckthorn Berries Attenuate Pulmonary Vascular Hyperpermeability in Lipopolysaccharide-induced Acute Lung Injury in Mice

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Abstract

Background Acute lung injury is featured by pulmonary vascular hyperpermeability, resulting in high short-term mortality. Currently pharmacological therapies are still sparse. Methods In the mice model of acute lung injury induced by Lipopolysaccharide, the effect of seabuckthorn berries extract on pulmonary vascular hyperpermeability was evaluated by histopathologic observation and transvascular leakage determination. The key factors involved in alveolar-capillary barrier lesion were assessed. Results The findings indicated that treatment of seabuckthorn berries alleviated morphological lesion as well as water, Evans blue and total proteins leakage in lung tissue, suppressed the release of TNF-α and IL-6, decreased accumulation of neutrophils, inhibited the activation of NF-κB and down-regulated the expression of ICAM-1 and CD62E. Conclusions These results demonstrated seabuckthorn berries help maintaining alveolar-capillary barrier integrity under endotoxin challenge in mice by suppressing the key factors in the pathogenesis of acute lung injury.

Background

Acute lung injury (ALI) is a clinical syndrome characterized by progressive hypoxemia and respiratory distress, caused by diverse endogenous and exogenous factors that injure lung directly or indirectly. ALI is the source of substantial morbidity and mortality in both adult [1, 2] and pediatric [3, 4] populations and is a major contributor to intensive care unit (ICU) costs [5]. Sepsis, induced by severe infection of bacteria, virus or fungus, is one of the leading etiologies of ALI [6]. It may cause sequencial functional disorders to multiple organs, among which ALI occurs at the earliest stage and with the highest morbidity [7, 8]. Long term clinical observations and experiments have demonstrated that ALI is the distinct manifestation of systemic inflammatory response syndrome (SIRS) in lung. Severe inflammatory response and protein-rich edema fluids induced by vascular endothelial injury are presented through the entire process [9]. Under pathogen stress, monocyte/macrophage system is stimulated through Nuclear Factor-kappa B (NF-κB) signal pathway to release pro-inflammatory cytokines such as Tumor Necrosis Factor alpha (TNF-α) and Interleukin-6 (IL-6), which in turn mediate adhesion and combination of neutrophils to endothelial cells in pulmonary microvessels by regulation of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin (CD62E) [10, 11]. Activation of neutrophils results in the release of mediators (e.g., oxidants and proteases) that increase vascular permeability by disrupting interendothelial junctions, thereby intravascular fluid and macromolecules permeate into alveoli and cause edema formation in pulmonary tissue.

Pharmacological therapies are still sparse for ALI, and supportive treatment of low tidal volume ventilation is the main strategy in clinical practice [12]. However, mechanical tension generated by ventilation itself may introduce inflammatory response and cause further injury [13]. Therefore, searching for alternative therapeutic strategy is of substantial interests.

Seabuckthorn (Hippophae rhamnoides L., Elaegnaceae) berries have been used to treat lung diseases in traditional Tibetan medicine for a long history. Modern research revealed that the berries contain various

constituents, including vitamins, fatty acids, free amino acids, carotenoids and phenolic compounds, and present remarkable anti-oxidative and anti-inflammatory activities [14]. In the current work, we investigated the effect of seabuckthorn berries on Lipopolysaccharide (LPS)-induced ALI in mice and explored the possible mechanism.

Materials And Methods

Chemicals and reagents

Lipopolysaccharide (O55:B5) was purchased from Sigma (MO, USA). BCA, TNF-α and IL-6 ELISA kits were purchased from Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-NF-κB (p65), rabbit anti-IKK, mouse anti-ICAM-1, rabbit anti-CD62E antibodies and 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride were purchase from Abcam (MA, USA). The nuclear extract kit was purchase from Active Motif (CA, USA). The PVDF membrane was purchase from Millipore (MA, USA).

Plant material and extract preparation

Well-ripened seabuckthorn berries were collected from natural growth site of hilly region in eastern margin of Tibetan Plateau (Ma'erkang, Sichuan province, China) and identified as *Hippophae rhamnoides* L., Elaegnaceae. Voucher specimens (No. MZC-SJ-20180924-00~03) of the plant material are preserved in the herbarium of Chengdu University of Traditional Chinese Medicine after botanical identification.

The berries were refluxed with deionized water and the supernatant was concentrated under vacuum to yield seabuckthorn berries extract (SBE). We analyzed chemical constituents and established HPLC chromatographic fingerprint spectrum of seabuckthorn berries in previous work [15].

Animals

Since the most common cause of ALI in humans is sepsis, the administration of gram-negative bacteria endotoxin, lipopolysaccharide, has been widely used as an animal model of sepsis-related lung injury in several species [16]. The present study was performed in 6 batches of male KM mice each weighing 18–22 g maintained at 24 ± 0.5℃ with food and water *ad libitum*. The experimental was approved by the Institute's animal ethical committee and confirmed to national guidelines on the use and care of laboratory animals.

After acclimatization for 2 days, mice were randomly allocated into control group, LPS group and three SBE groups of different dose levels (120, 240 and 480 mg/kg•bw, respectively). Animals in each group received respective treatment (corresponding doses of SBE for SBE groups and saline for control group) once daily through intragastric route for seven consecutive days. On Day 8, mice were administered saline for control group and LPS (O55:B5) for other groups by intraperitoneal injection at a dose of 10 mg/kg. Animals were sacrificed 10h after injection.

Gross and histopathologic observation

After the animal was sacrificed, median sternotomy was performed to expose trachea and pleuroperitoneal cavity, so that lung was excised for visual inspection. Then the lung was fixed with an intratracheal instillation of 1 ml buffered formalin (10 %, pH 7.2). The lobe was further fixed in 10 % neutral buffered formalin for 48 h at 4 °C. The tissues were embedded in paraffin wax. Sections approximately 5 μm thick were stained with hematoxylin and eosin using a standard protocol and observed under the light microscope for histopathological changes such as alveolar septum lesion, inflammatory cell infiltration, blood stasis, etc.

Lung water content determination

Wet-to-dry weight ratio was used as an index to estimate the degree of pulmonary edema. After the animal was sacrificed, lungs were excised enbloc, blot dried and placed on pre-weighed glass plates. The wet weight of the tissue was registered immediately. Then the tissue was placed in an incubator at 80 °C for 72 h to obtain a constant weight. After the dry weight of the tissue was registered, the water content of the tissue was calculated as wet weight/dry weight ratio (W/D).

Transvascular leakage analysis

In order to evaluate LPS-induced lung vascular leak, 1% Evans blue dye in saline (10 ml/kg, Sigma, USA) was injected into the tail vein one hour before termination of the experiment. Measurement of Evans blue accumulation in the lung tissue was performed by spectrofluorimetric analysis of lung tissue lysates according to the protocol described previously [17, 18].

Protein content in bronchoalveolar lavage fluid (BALF) reflects macromolecule leakage through impaired endothelia barrier. To analyze BALF, animal's trachea was exposed and an intravenous infusion needle was inserted. The lungs were lavaged three times with 0.5 ml of ice-cold phosphate-buffered saline. Returned lavage fluid was pooled for each animal and centrifuged at 800×g for 5 min at 4°C. The supernatants were harvested for total protein analysis using BCA protein assay kit and the sediments were collected for neutrophils count under light microscope on cytospin slides stained with Wright's solution.

Inflammatory cytokines assay

To evaluate inflammatory response, blood samples from the abdominal aorta of mice were collected before the animals were sacrificed, followed by incubation for 1 h under 37°C and centrifugation for 5 min at 1500×g. Serum cytokines TNF-α and IL-6 levels were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

Immunofluorescent analysis

The transcription factor NF-κB serves as a pivotal mediator of inflammatory response. Activation and nuclear translocation of NF-κB induces various pro-inflammatory cytokines. The expression of NF-κB p65 in lung cells was determined by immunofluorescence technique. Sections of lung tissue were deparaffinized and rehydrated through submersion in graded alcohols. Antigen retrieval was performed with 10 mM citrate buffer pH 6, for 5 min in a microwave oven. The sections were incubated with a primary antibody against NF-κB p65 (1:400), followed by detection with a fluorescein-conjugated secondary antibody (1:100). The nuclei were counterstained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI). The fluorescent images were captured using appropriate filters in a Nikon inverted fluorescent microscope (Tokoyo, Japan), and IOD (Integrated optical density) and PPA (Percent of positive area) for photomicrographs were calculated using image processing software Image-Pro Plus 6.0 (Media Cybernetics, USA).

Western blot

To evaluate the expression of proteins relative to NF-κB signal pathway, the contents of cytoplasmic Inhibitor of Nuclear Factor-κB Kinase (IKK) and nuclear P65, as well as downstream CD62E and ICAM-1, were determined using western blot technology. Lung tissue homogenate were centrifugation (12,000×g, 10 min, 4 °C) and supernatants were aspirated. Biochemical fractionation of the cells was done using the nuclear extract kit according to the manufacturer's instructions. Proteins were loaded and transferred to a PVDF membrane. After being blocked, membranes were incubated overnight at 4°C with a primary antibody, followed by incubation with secondary antibody for 1 h at room temperature. The membranes were placed into a gel imaging system (Bio-Rad, ChemiDoc XRS, USA) and then exposed. The intensity of blots was quantified using the Quantity One Analysis software (Bio-Rad, USA).

Statistical analysis

Data were expressed as mean ± standard deviation. All statistical analysis was performed with Prism 8 software (GraphPad Software, CA, USA). Statistically significant differences between groups were determined by ANOVA followed by Tukey's test. Results were considered statistically significant if P values were <0.05.

Results

Morphological Changes

Figure 1 shows the morphological differences between the lungs of animals from Control, Model and SBE groups. Compared with healthy tissue of Control group, LPS stimulation caused obvious lesion in lungs of model animals, including foamy mucus in some tracheas, enlarged lobes, darker in color with scattered petechiae, and incisions exudation. While SBE treatment provided protection to a certain degree, with alleviated edematous lesion and fewer petechiae observed.

Effects of SBE on vascular permeability

The stimulation of LPS caused edema in lung tissue, reflected in a remarkable increase of Wet-to-dry weight ratio (5.13 ± 0.19 vs. 4.69 ± 0.22). Pretreatment with SBE reversed the increase in a dose-dependent manner, with significant difference in SBE 480mg/kg group (Figure 2a). The result indicated that SBE alleviated edema in lung tissue induced by LPS.

Evans blue accumulation in lung tissue through transvascular leakage was remarkable higher in LPS group than that in control group (4.58 ± 0.97 vs. 1.68 ± 0.47 μg/g). Protein concentration and neutrophils count in BALF both sharply increased in LPS group, compared to those in control group (1.98 ± 0.43 vs. 0.60 ± 0.37 μg/g, 20.04 ± 5.02 vs. 0.63 ± 0.74, respectively). These results suggested that LPS induced the increase in vascular permeability, resulting in transvascular leakage of dye and proteins. Pretreatment of SBE alleviated the endothelia barrier lesion in a dose-dependent manner, with both 240 and 480 mg/kg groups showing statistic significance (Figure 2a, 2b and 2c).

Effects of SBE on inflammatory cytokines

Cytokines TNF-α and IL-6 levels in serum were higher in LPS group than those in control group (489.71 ± 118.99 vs. 279.48 ± 105.90 pg/mL, 5.50 ± 0.16 vs. 4.78 ± 0.11 ng/mL, respectively), which suggested that LPS induced inflammatory response in mice. In SBE treatment groups, TNF-α and IL-6 levels both decreased compared to LPS group, with statistic significances in 480 mg/kg group for TNF-α and all three dose groups for IL-6 (Figure 3).

Effects of SBE on NF-κB activation

The immunofluorescence images showed enrichment of NF-κB P65 protein both in the nuclear and cytoplasmic fraction of lung cells in LPS group, with green fluorescence for NF-κB P65 protein and blue fluorescence for nuclear. It suggested the increased expression and nuclear translocation of NF-κB upon LPS stimulation. In SBE 480 mg/kg group, fluorescence intensity appeared weaker than that of LPS group. Furthermore, IOD and PPA assays both indicated SBE treatment reversed the increased immunofluorescence intensity induced by LPS, with statistic significance in 480 mg/kg group (Figure 4).

The results of western blot showed that low basal expression levels of cytoplasmic IKK and nuclear P65 increased significantly upon LPS stimulation (0.41 ± 0.02 vs. 0.11 ± 0.02 and 0.50± 0.03 vs. 0.09 ± 0.02, respectively), suggesting the release and nuclear import of NF-κB. By contrast, the expression of IKK and P65 decreased in a dose-dependent manner in SBE groups with statistic significance at each dose level (Figure 5). Similarly, treatment of SBE reserved LPS-induced increased expression of two downstream proteins of NF-κB, ICAM-1 and CD62E (Figure 6). These results demonstrated that SBE inhibited the activation of NF-κB induced by LPS.

Discussion

In the present study, we investigated the effect of seabuckthorn berries on ALI in mice, inspired by its therapeutic use in traditional Tibetan medicine for treating various pulmonary diseases and relieving

hypoxic respiratory distress. Since sepsis is the most common clinical setting in which ALI develops and bacterial endotoxin is implicated as an important toxin precipitating lung injury, the widely-accepted sepsis-related lung injury model by LPS administration was used. Anotomical findings revealed severe pulmonary tissue injuries induced by LPS, including foamy mucus and scattered petechiae, were alleviated to some extent by SBE treatment.

Airway vascular endothelial injury is a major pathological feature of ALI. Endotoxin induces inflammatory response, with accumulation of inflammatory mediators in lung tissue, causing alveolar-capillary barrier lesion, which is associated with increased vascular permeability and accumulation of protein-rich interstitial and alveolar fluid. We determined lung water content by wet-to-dry weight ratio, which increased obviously in LPS group, indicating pulmonary edema in model animals. Albumin leakage was determined by Evans blue assay. Intravenously administrated Evans blue binds to serum albumin with high affinity and serves as a probe to trace albumin leakage [19]. Elevated level of Evans blue concentration in lung tissue of LPS group showed pulmonary vascular hyperpermeability. This is verified by increased total protein concentration in BALF of LPS group. These findings demonstrated the integrity of alveolar-capillary barrier was impaired in LPS-induced ALI mice, consistent with other reports [12, 16, 17]. By contrast, data in SBE groups showed alleviation of transvascular leakage in a dose-dependent manner. These results suggest that seabuckthorn berries can protect alveolar-capillary barrier integrity upon endotoxin challenge.

Activation of neutrophils sequestered in pulmonary microvessels is an important factor in the pathogenesis of increased lung vascular permeability and tissue injury [20]. We found in this study that elevated level of neutrophils in BALF induced by LPS was reversed by SBE treatment, which is consistent with the endothelial permeability results.

Inflammation is associated with the pathological process of ALI. Pro-inflammatory cytokines TNF-α and IL-6 have been strongly implicated in the pathogenesis of ALI in human and animal models [21]. Our results confirmed that SBE curtailed TNF-α and IL-6 release induced by LPS, showing significant anti-inflammatory activity.

Since NF-κB signal pathway plays a key role in inflammatory response, we assume it may be the effect target of SBE. In a quiescent state, NF-κB dimers are anchored in the cytoplasm by inhibitor IκB. When activated by signals, IKK degrades IκB through phosphorylation and ubiquitination, and NF-κB is then freed to enter the nucleus where it can turn on the expression of relative genes. We investigated NF-κB expression in lung tissue by immunofluorescent analysis, as well as cytoplasmic IKK and nuclear NF-κB P65 expression by Western blot. The results demonstrated that SBE suppressed the expression of IKK and the nuclear translocation of NF-κB stimulated by LPS. The downstream proteins of NF-κB relative to neutrophil activation include ICAM-1 and CD62E, which mediate neutrophil interaction with endothelial cells. We also evaluated the expression levels of the two proteins and confirmed the similar suppression by SBE. These findings provide consistent evidences supporting the inhibition activity of SBE on NF-κB signal pathway.

The notable alveolar-capillary barrier protection and anti-inflammtory activity of seabuckthorn berries may attribute to multiple constituents. The main active ingredients include flavonoids, a kind of natural anti-oxidative and anti-inflammtory agents. Total flavonoids from seabuckthorn have been used in treating cardiovascular disease. Also, the high content of vitamins (B, C, E and K) makes seabuckthorn a popular nutritional supplement. Oil from seabukchtorn berries containing fatty acids and carotenoids facilitates the wound healing in burning and ulcer. Therefore, SBE may serve as a natural complex preparation to provide beneficial effect in ALI mice. The specific contribution for individual component merits further investigation.

Conclusion

In the present research, we demonstrated that seabuckthorn berries can protect alveolar-capillary barrier from hyperpermeability in LPS-induced ALI mice by suppressing the key factors in the pathogenesis of ALI, including the release of cytokines TNF-α and IL-6, the activation of NF-κB signal pathway, the expression of ICAM-1 and CD62E, and the adhesion of neutrophil to endothelial cell. The effective constituents need further research and development, in order to provide a supplemental preventive and therapeutic strategy for ALI.

Abbreviations

ALI : Acute lung injury; SIRS: inflammatory response syndrome; NF-κB: Nuclear Factor-kappa B; TNF-α: Tumor Necrosis Factor alpha; IL-6: Interleukin-6; ICAM-1: intercellular adhesion molecule-1; CD62E: E-selectin; LPS: Lipopolysaccharide; SBE: seabuckthorn berries extract; BALF: bronchoalveolar lavage fluid; ELISA: enzyme-linked immunosorbent assay; IOD: Integrated optical density; PPA: Percent of positive area; IKK: Nuclear Factor-κB Kinase.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publication

Not applicable.

Availability of data and materials

The research data generated from this study is included within the article.

Competing interests

The authors declare that they do not have any conflicts of interest.

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Authors' contributions

C. Chen and G. Fan designed research; L. Du, Y. Liu, and L. Wan performed research; C. Chen and Y. Liu collected seabuckthorn samples and identified the specimen; L. Du and G. Fan wrote the paper.

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Not applicable.

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Figures

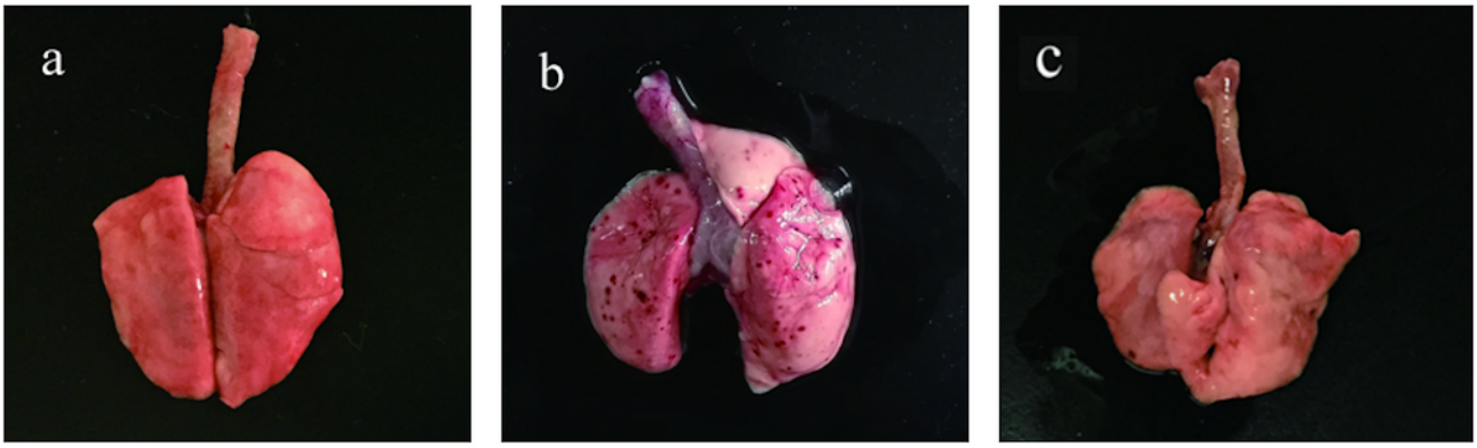

Figure 1

Effect of lipopolysaccharide on lung morphology. (a) Control group. (b) Model group. (c) SBE 480mg/kg group.

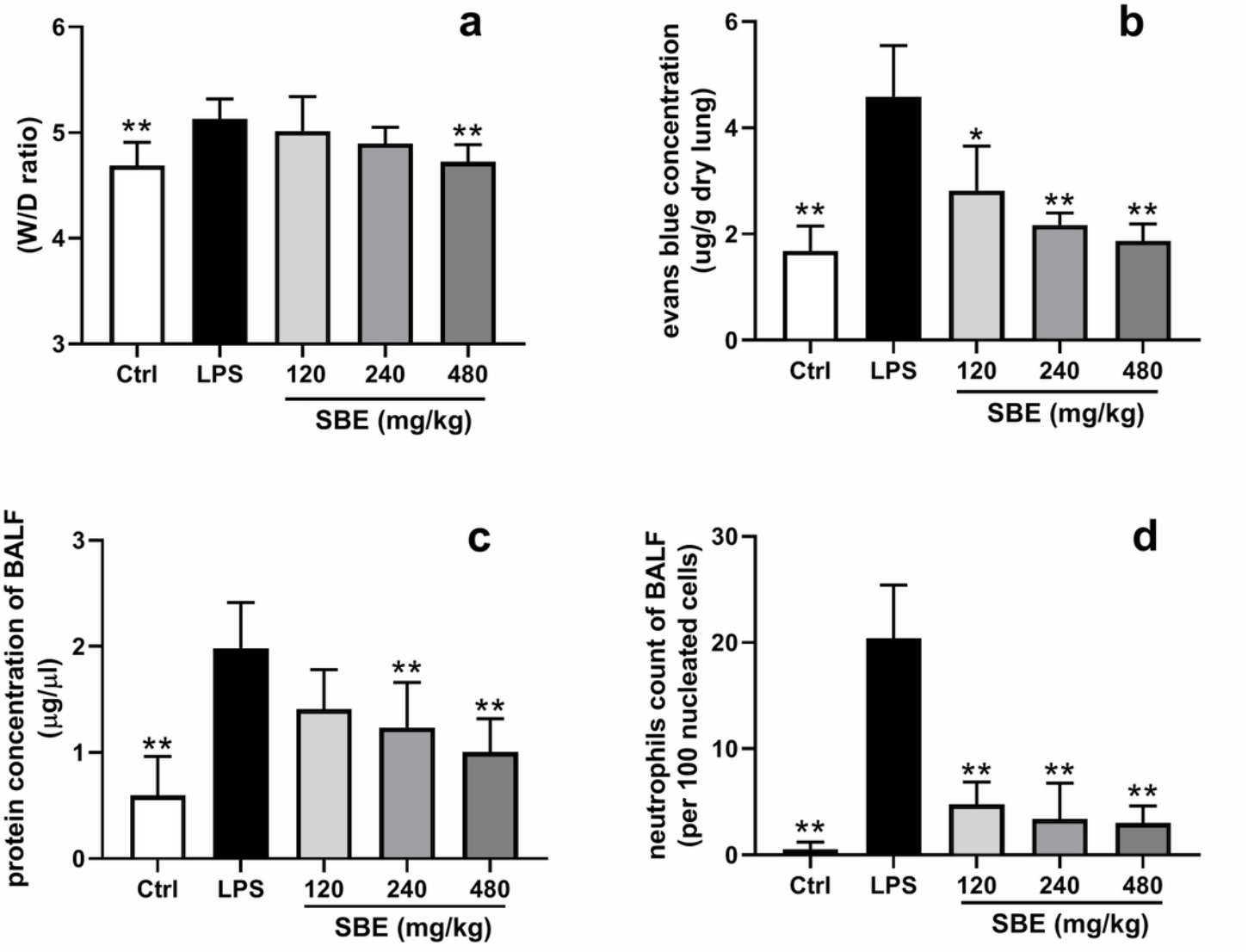

Figure 3

Effects of SBE on vascular permeability, evaluated by (a) wet-to-dry ratio, (b) pulmonary vascular leakage, and (c) total protein and (d) neutrophils concentration in BALF. The results were presented as mean ± SD (n = 10). *p < 0.05 or **p < 0.01, vs. LPS group.

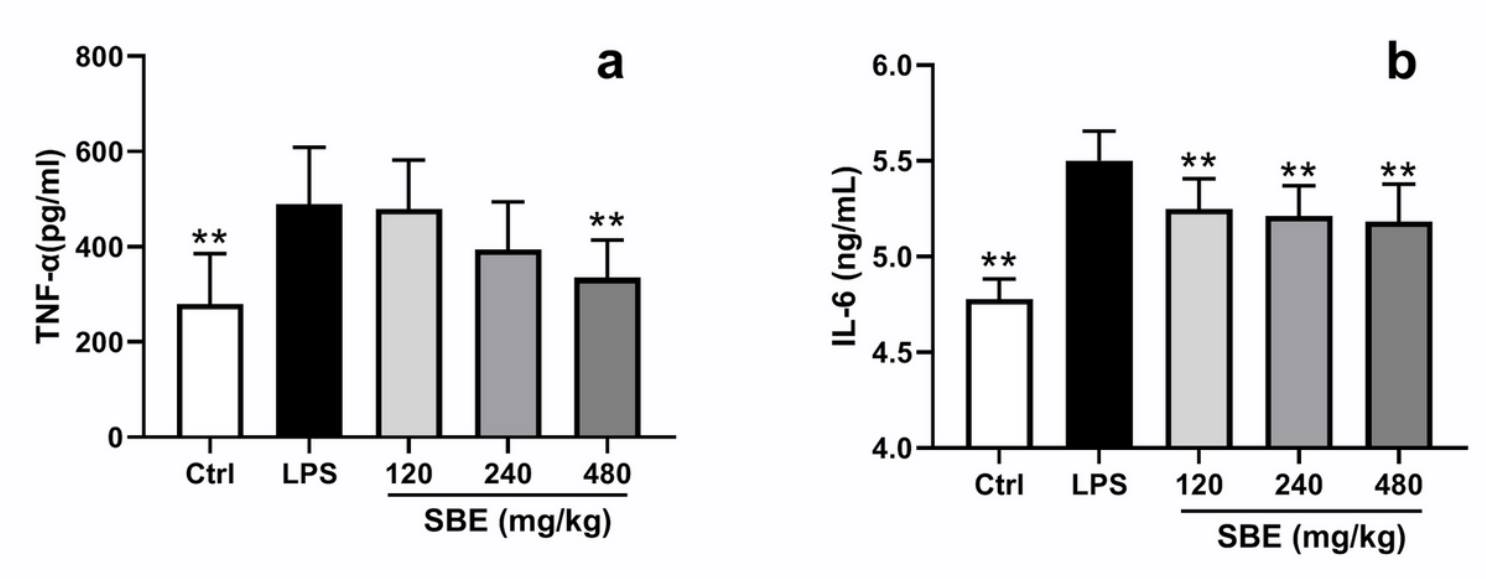

Figure 5

Effects of SBE on cytokines (a) TNF-α, and (b) IL-6. The results were presented as mean ± SD (n = 10). **p < 0.01, vs. LPS group.

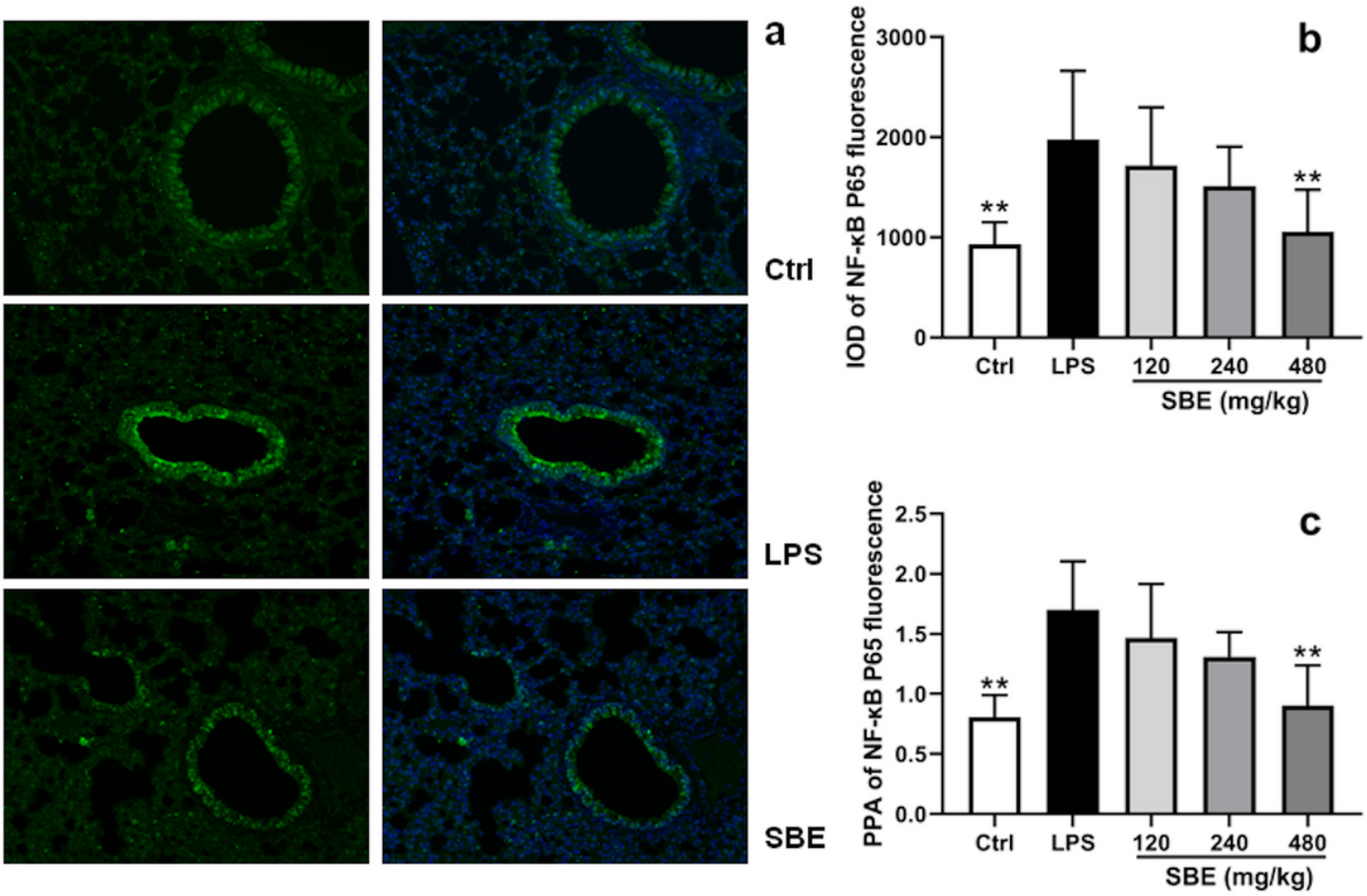

Figure 7

Effects of SBE on NF-κB P65 immunofluorescence (a) immunofluorescence images (control, LPS and SBE 480 mg/kg group, respectively), (b) IOD and (c) PPA analysis of fluorescence intensity. The results were presented as mean ± SD (n = 10). **p < 0.01, vs. LPS group.

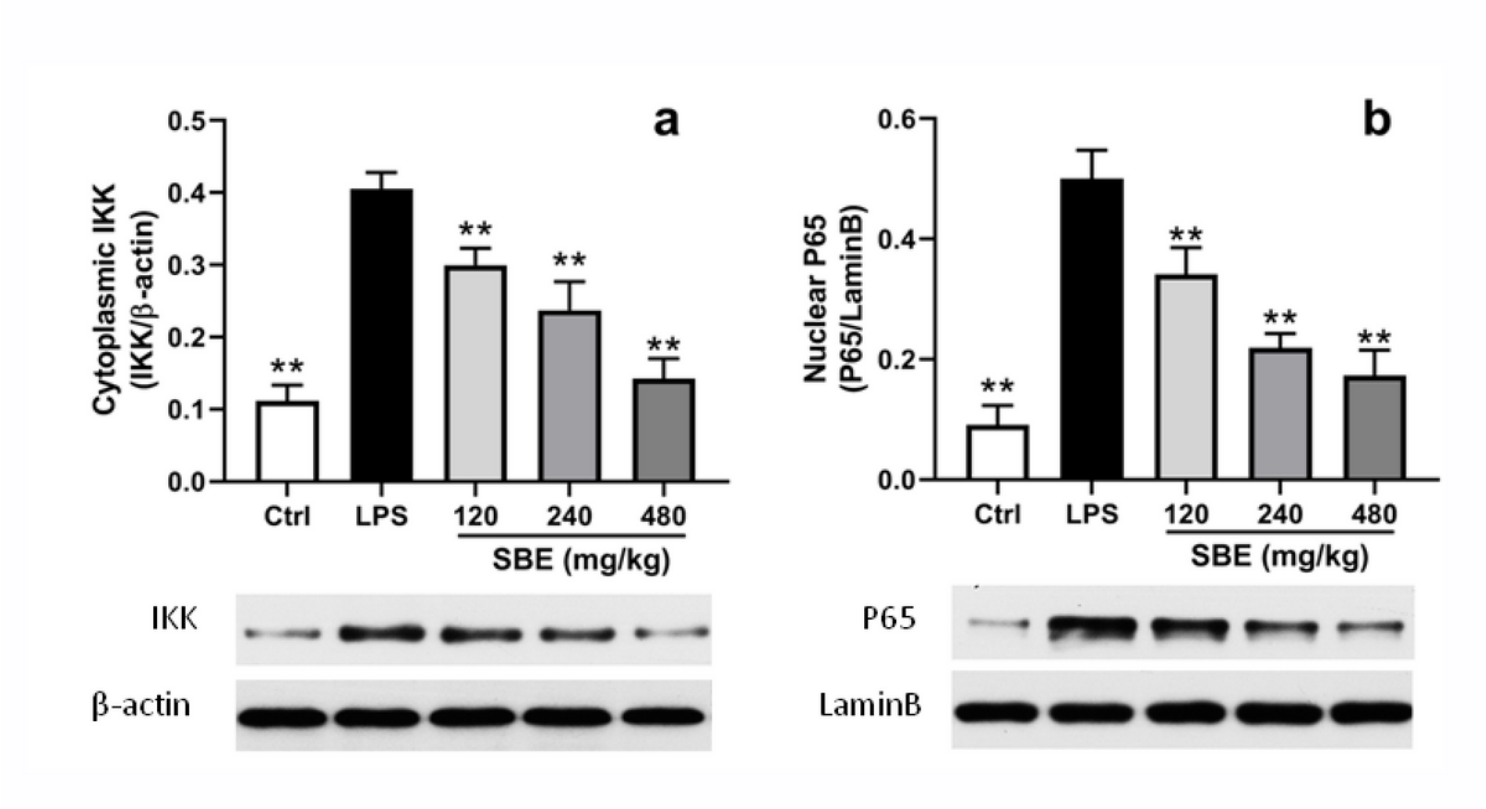

Figure 9

Effects of SBE on expression of (a) cytoplasmic IKK and (b) nuclear P65. The results were presented as mean ± SD (n = 6). **p < 0.01, vs. LPS group.

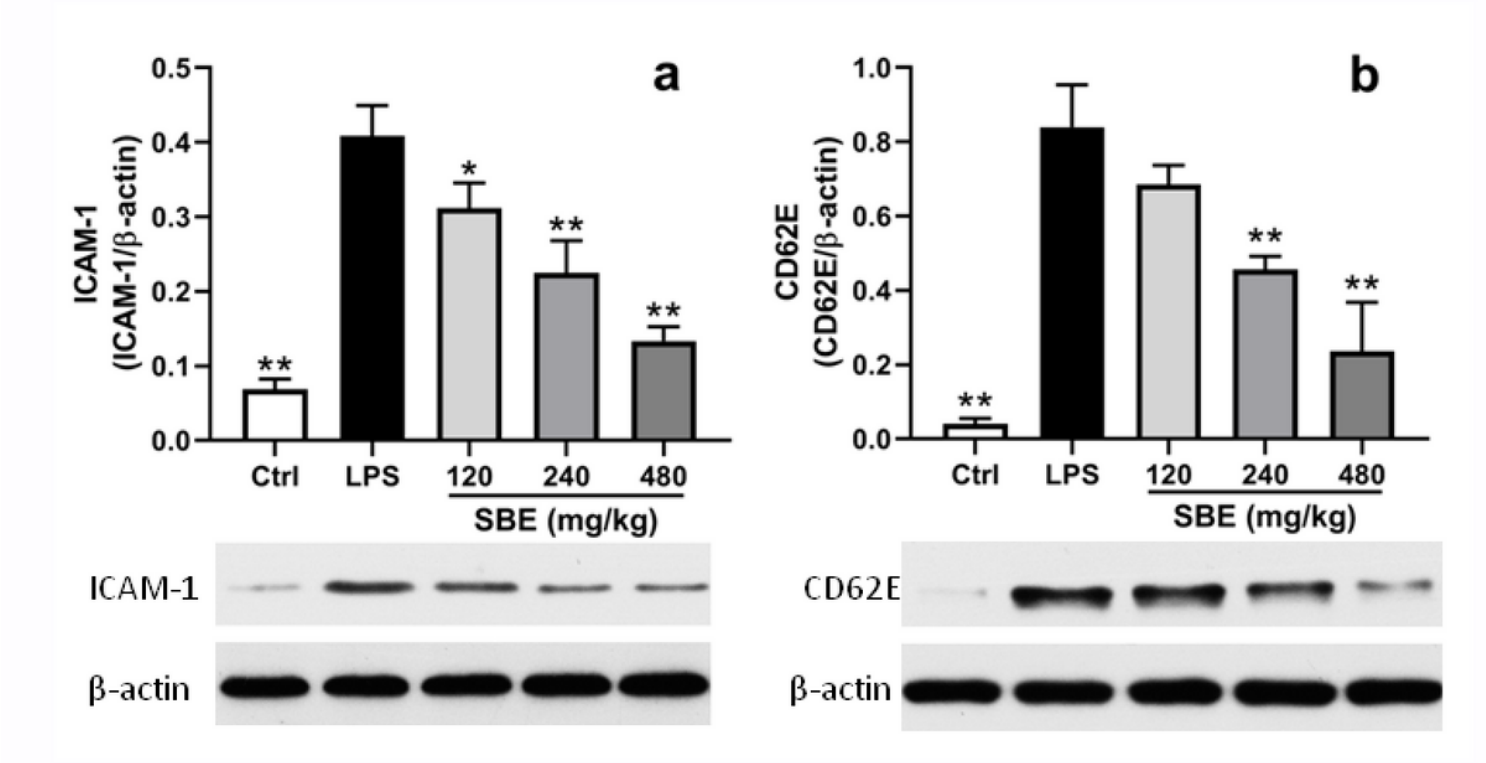

Figure 12

Effects of SBE on expression of (a) ICAM-1 and (b) CD62E. The results were presented as mean ± SD (n = 6). *p < 0.05 or **p < 0.01, vs. LPS group.