Original Research

Grape seed proanthocyanidin extract alleviates arsenic-induced lung damage through NF-κB signaling

Yunhua Hu[1,*], Meng Wei[1,2,*], Qiang Niu[1], Rulin Ma[1], Yu Li[1], Xianhua Wang[3], Gangling Feng[1], Shugang Li[1] and Lijuan Pang[1]

[1]Department of Public Health, Shihezi University School of Medicine, Shihezi 832003, China; [2]Department of Outpatient, The First Affiliated Hospital, Xinjiang Medical University, Xinjiang 830054, China; [3]Department of Quality Control of Changji Hui Autonomous Prefecture Center for Disease Control and Prevention in the Xinjiang Uygur Autonomous Region, Changji, Xinjiang 831100, China;
*These authors contributed equally to this work.
Corresponding author: Shugang Li. Email: lishugang@ymail.com

Impact statement

Arsenic-induced respiratory inflammatory damage is an important occupational hazard in many areas of the world, particularly in underdeveloped and developing countries. Effective treatments are lacking and expensive. Therefore, the aim of the study was to examine the anti-inflammatory effects of proanthocyanidin (PC) and the molecular mechanisms in vivo and in vitro. The present study showed that PC extracted from grape seed could attenuate the lung damage in a mouse model of arsenic poisoning. The effects were observed at the level of lung histology and inflammasome expression. This study suggests that a natural compound is effective in mitigating the toxic effects of arsenic in the lungs, providing an inexpensive and more readily accessible method for treating arsenic exposure in some parts of the world.

Abstract

Proanthocyanidin has beneficial features such as free radical scavenging, anti-inflammation, and anti-oxidation. There is no study on whether proanthocyanidin could protect against arsenic-induced respiratory inflammatory damage. The aim of the study is to examine the anti-inflammatory effects of proanthocyanidin and the molecular mechanisms in vivo and in vitro. BEAS-2B cells were treated with As_2O_3, grape seed proanthocyanidin extract (GSPE), and/or BAY 11–7082. Kunming mice were treated with As_2O_3 and/or GSPE. p-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 were assessed by Western blot and qRT-PCR. Lung histology was examined. Arsenic affected the histology of the mouse lungs, but GSPE attenuated those effects. As_2O_3 increased cell apoptosis, which was reversed by GSPE. In cells and mouse lung tissue, arsenic increased the expression of IL-1β, IL-6, tumor necrosis factor-α, and C-reactive protein, and these effects were attenuated by GSPE. In cells and mouse lung tissue, arsenic enhanced the mRNA and protein levels of IKKα, IKKβ, NF-κBp65, and NF-κBp50, while the IκB-α levels were decreased compared with controls. IKKα, IKKβ, NF-κBp65, and NF-κBp50 mRNA and protein levels in the As_2O_3+GSPE groups were lower and IκB-α levels were higher than that in the arsenic groups. Arsenic-activated NF-κB signaling induced inflammatory damage through the upregulation of pro-inflammatory cytokines and downregulation of anti-inflammatory cytokines. GSPE plays a beneficial role against arsenic-induced inflammatory damage through, at least in part, the suppression of the arsenic-induced NF-κB signaling pathway.

Keywords: Arsenic, lung injury, nuclear factor κB, proanthocyanidin

***Experimental Biology and Medicine* 2019; 244: 213–226. DOI: 10.1177/1535370219829881**

Introduction

Exposure to arsenic can cause significant respiratory damage.[1] Workers exposed to arsenic by inhaling dust and particles suffer from faucitis and bronchitis.[2] The acute exposure to arsenic also leads to respiratory syndrome.[3] Dutta et al.[4] assessed the sputum samples from 267 Indian women exposed to arsenic through drinking water and found that the number of inflammatory cells and the levels of tumor necrosis factor (TNF)-α, interleukin (IL)-8, IL-6, IL-12, and C-reactive protein (CRP) were significantly increased. Exposure to arsenic can affect the immune system by altering immune-related gene expression and cytokine secretion by lymphocytes.[5] In addition, a number of hormones are dysregulated, including retinoic acid, thyroid hormones, and estrogen receptors.[6] Diseases associated with low-grade inflammation (such as type 2 diabetes mellitus and chronic kidney disease) are also associated with chronic exposure to arsenic.[7] Many animal

models are available for studying the effects of arsenic,[8] as well as cell lines.[9] Among these, the BEAS-2B cell line has been shown to be an appropriate in vitro model to study the effects of heavy metals on human bronchial epithelium.[10]

The nuclear factor κB (NF-κB) is a protein complex that is central to transcription control of inflammatory genes, cytokine production, and cell survival. NF-κB is found in all animal cell types and is involved in cellular responses to stress, cytokines, free radicals, heavy metals, radiations, and microbe antigens.[11] Incorrect NF-κB regulation is associated with cancer, inflammatory diseases, autoimmune diseases, and incorrect immune development.[11] NF-κB plays a crucial role in arsenic-induced damage.[12] Arsenic induces the activity of IKK[13] and promotes the degradation of IκB.[14] The heterodimers of NF-κBp65 and NF-κBp50 are dissociated[15] and combine with the DNA binding site of κB.[16] The downstream genes related to inflammation are then activated, promoting the expression of inflammatory factors that contribute to inflammatory damage.[17]

Proanthocyanidin (PC) is a polyphenolic compound that is present at high concentration in the nucleus, pericarp, and seed of grapes.[18] PC has beneficial features such as free radical elimination, anti-inflammatory, and anti-oxidation.[19–21] A previous study showed that PC prevented endothelial dysfunction by inhibiting NF-κB.[19] Limtrakul et al.[21] showed that PC inhibited inflammation through the suppression of the AP-1, NF-κB, and MAPK pathways in Raw 264.7 macrophages. PC has been shown to protect against lung inflammation and remodeling in mouse models of lung inflammation through downregulation of inflammatory cytokines.[20] Nevertheless, to the best of our knowledge, there is no study on whether PC could protect against arsenic-induced respiratory inflammatory damage and whether the molecular mechanism of the eventual protective effect could be associated with NF-κB signaling.

BAY 11–7082 is a NF-κB inhibitor that has been shown to inhibit the NLRP3 inflammasome and to decrease the pro-inflammatory cytokines IL-1β and IL-18 that participate in chronic inflammatory diseases.[22] Lappas et al.[23] showed that BAY 11–7082 reduced IL-6, Il-8, and TNF-α secretion by adipocytes. In cancer cells, NF-κB is involved in cell survival, and BAY 11–7082 has been shown to decrease the survival of those cells by inhibiting NF-κB.[24] BAY 11–7082 has been shown to decrease inflammatory markers in cell lines treated with arsenic,[25] indicating the usefulness of BAY 11–7082 to study the effects of arsenic.

Therefore, the present study examined the anti-inflammatory effects of PC and the molecular mechanisms in vivo and in vitro. The results could provide a reference for the treatment of arsenic poisoning and the development and utilization of PC.

Materials and methods

Cell culture and treatment

Human bronchial epithelial cells (BEAS-2B cells) were obtained from Shanghai FuHeng Biology Co. (Shanghai, China). The BEAS-2B cells were cultured in Dulbecco's modified eagle medium (DMEM; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific, Waltham, MA, USA), and penicillin–streptomycin mixture (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under 5% CO_2. The cells were treated with As_2O_3 (0, 5, 10, or 20 μmol/L in 4 M NaOH solution) (#890314; Beijing Chemical Plant, Beijing, China) and grape seed proanthocyanidin extract (GSPE; 0, 25, and 50 mg/L in deionized water; Tianjin Jianfeng Natural Plants Company, Tianjin, China; >95.0% purity) for 24 or 48 h, according to a previous study.[26] BAY 11–7082 was bought from Abcam (Cambridge, United Kingdom; ab141228; >99% purity) and used at 50 mg/L in 0.1% dimethyl sulfoxide (DMSO) (Sigma, St Louis, MO, USA).

MTT assay

BEAS-2B cells in the logarithmic growth phase were inoculated in 96-well culture plates at 5000 cells/well in a total volume of 200 μL/well. All experiments were performed in triplicates (three parallel wells). MTT (Nanjing Jiancheng Inc., Nanjing, China) and DMSO (Sigma, St Louis, MI, USA) were used to detect the viability of the cells using a microplate spectrophotometer to measure the 490-nm OD (reference OD of 620 nm) in each well.[27]

Flow cytometry

BEAS-2B cells in the logarithmic growth phase were cultured; 1–3 × 10^6 cells were collected, resuspended in 500 μL of apoptosis positive control solution, and incubated on ice for 30 min. Cold binding buffer (1×; 1.0 mL) was added. The solution was divided equally into three: one was the blank control and the other two were staining tubes. Annexin V-FITC (5 μL; Tianjin Sungene Biotech Co., Ltd, Tianjin, China; AO2001-02P) was added into the two single staining tubes and incubated for 5 min with lucifuge. The samples were analyzed by flow cytometry to determine the apoptosis rates.[28]

Determination of reactive oxygen species and lipid peroxide

The Coomassie brilliant blue kit (A045-2, Nanjing Jiancheng Inc., Nanjing, China) was used to detect the total protein. The reactive oxygen species (ROS) kit (E004, Nanjing Jiancheng Inc., Nanjing, China) and lipid peroxide (LPO) kit (A106, Nanjing Jiancheng Inc., Nanjing, China) were used to measure the levels of ROS and LPO.[29]

Determination of inflammatory cytokines

ELISA kits were used to measure the levels of inflammatory cytokines in cell homogenates. IL-1β (L151112469, Cloud-Clone Corp, Wuhan, China), IL-6 (L151124780, Cloud-Clone Corp, Wuhan, China), IL-10 (L151109273, Cloud-Clone Corp, Wuhan, China), CRP (L151112436, Cloud-Clone Corp, Wuhan, China), and TNF-α (151124811, Cloud-Clone Corp, Wuhan, China) were detected. A microplate reader was used to read the plates at 450 nm.[30]

Western blot

The protein expression levels of the NF-κB signaling pathway (p-IκB-α (9246; Cell Signaling, Danvers, MA, USA), IκB-α (4814; Cell Signaling, Danvers, MA, USA), IKKα/β (ab178870; Abcam, Cambridge, MA, USA), NF-κBp65 (D14E12; Cell Signaling, Danvers, MA, USA), and NF-κBp50 (D4P4D; Cell Signaling, Danvers, MA, USA)) were detected by Western blot, as previously described.[31] β-actin (TA-09), HRP-labeled goat anti-rabbit IgG (H + L) (ZB-2301), and HRP-labeled goat anti-mouse IgG (H + L) (ZB-2305) were bought from ZSGB-BIO Inc. (Beijing, China). β-actin was used as a loading control.

Quantitative real-time PCR

According to Zhao and Zhang,[32] real-time PCR was used to detect the mRNA expression of IκB-α, IKKα, IKKβ, NF-κBp65, and NF-κBp50 from the cDNA samples. The primers were designed using Primer Premier 5.0 (Supplementary Table 1).

Animals

Forty healthy male Kunming mice were purchased from the Xinjiang Medical University Experimental Animal Center (license XK (Xin) 2011–00040). The mice were housed four/cage. Feed and water were provided ad libitum. A 2×2 factorial design protocol was designed using two factors and two levels: As_2O_3 (0 and 4 mg/kg) and GSPE (0 and 400 mg/kg), resulting in the control group, As_2O_3 group, GSPE group, and As_2O_3+GSPE group. There were 10 mice/group, randomly divided into each group with stratification for body weight. The control group was treated with 0.9% normal saline; the As_2O_3 group was administrated with 4 mg/kg As_2O_3; the GSPE group received 400 mg/kg GSPE; and the As_2O_3+GSPE received 4 mg/kg As_2O_3 and 400 mg/kg GSPE.

All groups were treated by intragastric administration for five weeks. The mice could eat and drink freely during the intervention. Based on the dose of 4.0 mg/kg and the total volume of the stomach is <20 mL/kg, the solution of As_2O_3 was 0.2 mg/mL, in water adjusted to be slightly alkaline, as previously described.[33] For GSPE, 4 g was dissolved in 100 mL of deionized water.

Lung tissue homogenate

Lung tissue homogenate was prepared as previously described.[34]

ROS and LPO in lung tissue

ROS and LPO were measured in lung tissue homogenate as previously described.[29]

Inflammatory factors in the lung tissue homogenate

IL-1β (L151214485), IL-6 (L151210437), IL-10 (L151221646), CRP (L151110355), and TNF-α (151102081) ELISA kits were bought from Cloud-Clone Corp. (Wuhan, China), and used as previously described.[30]

Western blot analysis of lung tissue

Western blot was performed as previously described[31] for the detection of p-IκB-α (9246; Cell Signaling, Danvers, MA, USA), IκB-α (4814; Cell Signaling, Danvers, MA, USA), IKKα/β (ab178870; Abcam, Cambridge, MA, USA), NF-κBp65 (D14E12; Cell Signaling, Danvers, MA, USA), and NF-κBp50 (D4P4D; Cell Signaling, Danvers, MA, USA). β-actin was used as a loading control.

Quantitative real-time PCR analysis of lung tissue

Lung tissue (100 mg) was grinded in liquid nitrogen, and added into 1 mL of TRIzol (Invitrogen Inc., Carlsbad, CA, USA). After 5 min, 200 μL chloroform was added and vortexed for 15 min. The solution was centrifuged for 15 min at 4°C and 12,000 $\times g$. The supernatant was collected for measuring the mRNA expression according to Zhao and Zhang.[32]

H&E staining of lung tissue

After sacrifice, the lungs were collected and part of the lung tissue was immersed in 10% formaldehyde for 24 h. The fixed lung tissue was embedded in paraffin and sectioned at 5 μm. The pathological changes of lung were observed after hematoxylin-eosin (H&E) staining.

Statistical analysis

The data were analyzed using SPSS 17.0 (Inc., Chicago, IL, USA). The data were expressed as means ± standard deviation. The factorial analysis of variance ($4 \times 3 \times 2 \times 2$) was used to display the main effects of As_2O_3, GSPE, BAY 11–7082, and time in vitro. The 2×2 factorial analysis was used to compare the main effect of As_2O_3 and GSPE in vivo. The interaction of As_2O_3 and GSPE was analyzed by the general linear model. The multiple comparisons among groups were performed using the Bonferroni test. $P < 0.05$ was regarded as statistically significant.

Results

Cell viability

A previous study showed that as the dosage of As_2O_3 increased, cell viability gradually decreased (from 100% to 65.3%) after 24 h.[35] A similar trend was also seen after 48 h (from 100% to 61.4%). When the same cells were treated with 50 mg/L GSPE, cell viability was 83.4% and 80.4%, respectively. When we applied BAY 11–7082 and GSPE at the same time, cell viability was 94.2% and 91.4%, respectively.

Effects of As2O3 and GSPE on apoptosis by flow cytometry

When cells were treated with As_2O_3 for 24 h, the apoptotic rate was increased (from 1.7% to 20.6%) and with increasing dose (Table 1, Figure 1(a) to (c)). For 48 h, the apoptotic rate was increased from 1.1% to 30.4%. After the addition of GSPE and BAY 11–7082, the apoptotic rate was decreased (Table 1, Figure 1(e) and (f)).

Table 1. Effects of As_2O_3 and GSPE on apoptosis by flow cytometry.

	Time	Dose of GSPE (mg/L)	Dose of As_2O_3			
			0 μmol/L (%)	5 μmol/L (%)	10 μmol/L (%)	20 μmol/L (%)
Without BAY 11-7082	24 h	0	1.7	7.2	20.1	20.6
		25	1.4	3.0	10.8	14.6
		50	2.3	1.8	7.2	12.3
	48 h	0	1.1	9.1	20.2	30.4
		25	1.4	5.5	11.2	16.2
		50	2.0	1.7	7.8	13.0
With BAY 11-7082	24 h	0	1.4	2.7	4.0	10.8
		25	1.7	2.3	4.2	6.3
		50	1.3	2.7	2.6	5.1
	48 h	0	1.1	2.6	8.7	14.9
		25	0.9	3.2	6.7	11.8
		50	1.4	3.1	4.2	10.0

GSPE: grape seed proanthocyanidin extract.

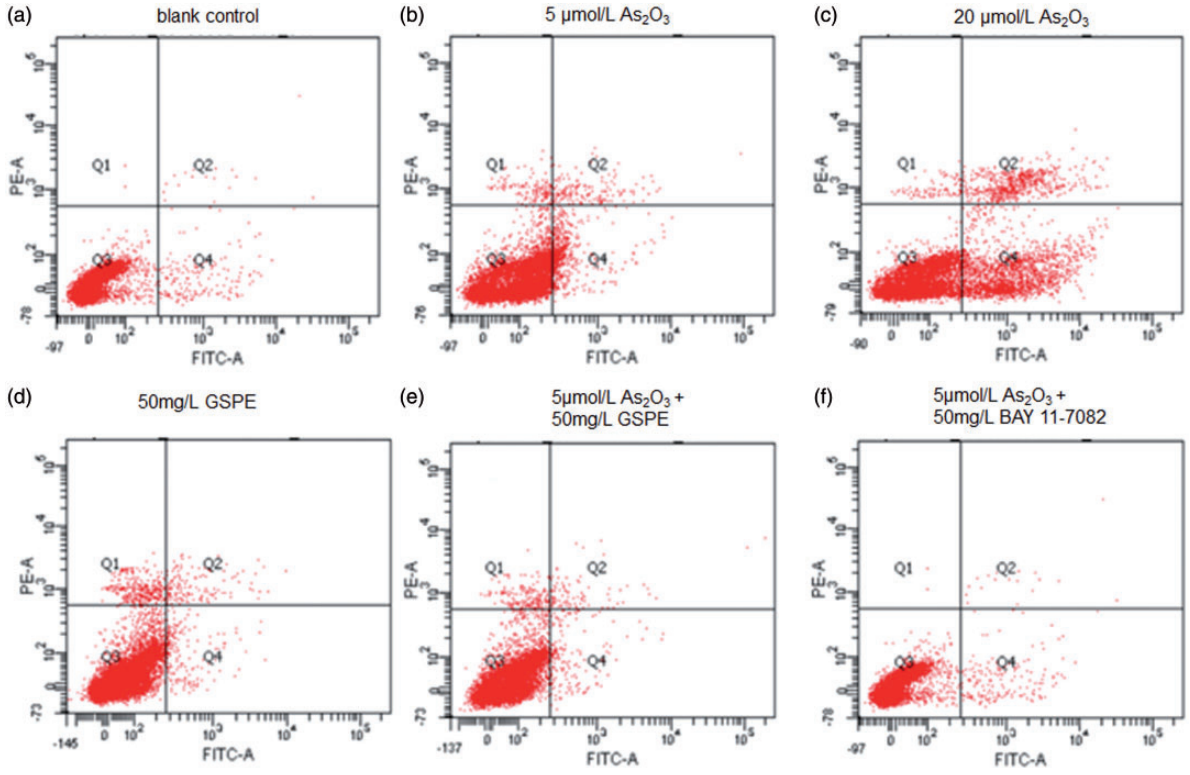

Figure 1. Effects of As_2O_3 and GSPE on apoptosis by flow cytometry. Apoptotic cells were quantified by flow cytometry. Q2 shows the apoptotic rate. (a) Blank control group (24 h), (b) 5 μmol/L As_2O_3 group (24 h), (c) 20 μmol/L As_2O_3 group (24 h), (d) 50 mg/L GSPE group (48 h), (e) 5 μmol/L As_2O_3 + 50 mg/L GSPE group (24 h), (f) 5 μmol/L As_2O_3 + 50mg/L BAY 11–7082 group (24 h). (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

Effects of As_2O_3, GSPE, and BAY 11–7082 on LPO and ROS in BEAS-2B cells

The concentrations of ROS and LPO decreased with the increasing GSPE concentration. A decreasing trend was shown in ROS and LPO when BAY 11–7082 was applied (Figure 2).

Effects of As_2O_3, GSPE and BAY 11–7082 on inflammatory cytokines of BEAS-2B cells

Exposure of BEAS-2B cells to arsenic for 24 and 48 h increased the expression of IL-1β, IL-6, TNF-α, and CRP in a dose-dependent manner while decreased IL-10 expression (Figure 3(a) to (j)). On the other hand, the levels of IL-1β, IL-6, TNF-α, and CRP were attenuated, and IL-10 was enhanced when different concentrations of GSPE and BAY 11–7082 were used (Figure 4(a) to (j)).

Effects of As_2O_3, GSPE, and BAY 11–7082 on mRNA expression of IκB-α, IKKα, IKKβ, NF-κBp65, and NF-κBp50 in BEAS-2B cells

Based on the results of cell apoptosis, viability, and inflammatory cytokines, we selected 5 μmol/L of As_2O_3 for

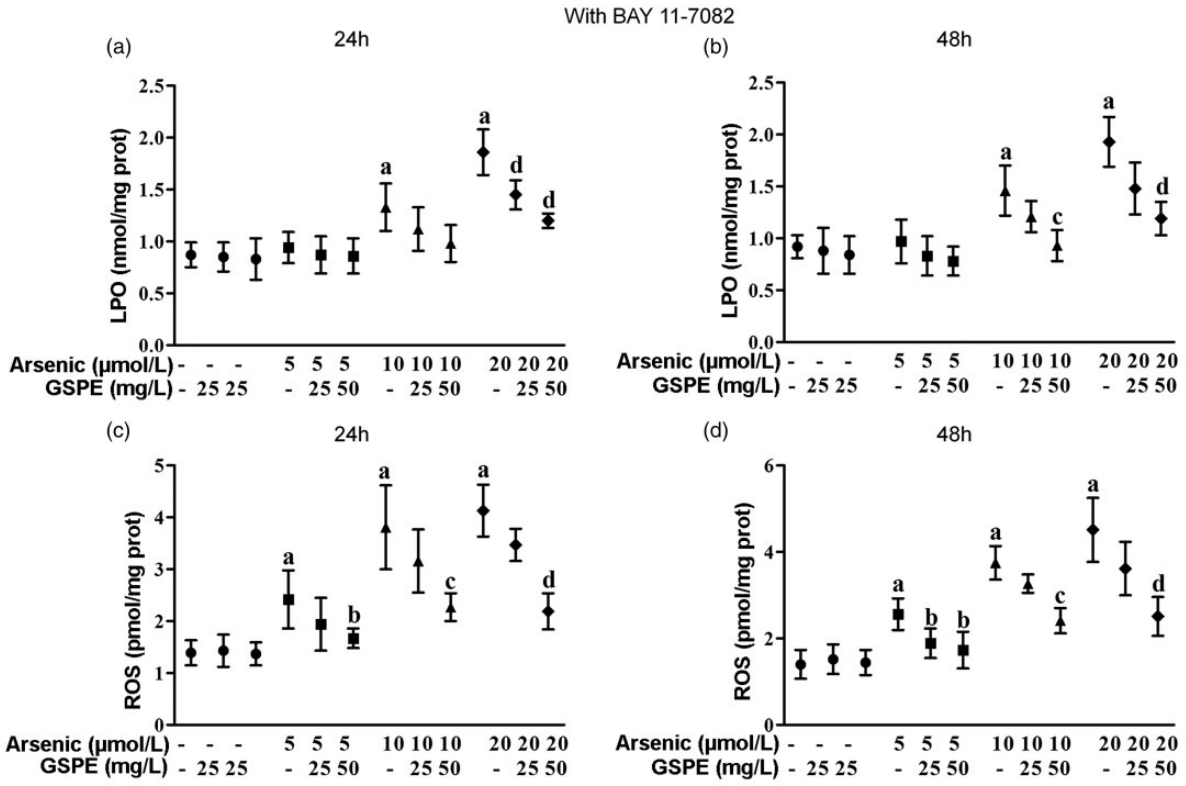

Figure 2. Effects of As_2O_3, GSPE, and BAY 11–7082 on LPO and ROS of BEAS-2B cells. The BEAS-2B cells were treated with As_2O_3 (0, 5, 10, or 20 μmol/L) (n = 3) and/or GSPE (0, 25, or 50 mg/L) (n = 3), all in combination with BAY 11–7082 (50 μmol/L). Values are expressed as means ± SD. $^aP < 0.05$, compared with blank control group; $^bP < 0.05$, compared with arsenic group (5 μmol/L); $^cP < 0.05$, compared with arsenic group (10 μmol/L); $^dP < 0.05$, compared with arsenic group (20 μmol/L). GSPE: grape seed proanthocyanidin extract; LPO: lipid peroxide.

exposure, 50 mg/L of GSPE, and 50 mg/L of BAY 11–7082 for intervention and 24 h for time. Arsenic treatment significantly enhanced the mRNA levels of IKKα, IKKβ, NF-κBp65, and NF-κBp50, while the IκB-α mRNA levels were decreased significantly compared with control. IKKα, IKKβ, NF-κBp65, and NF-κBp50 mRNA levels in the As_2O_3+GSPE group were lower and IκB-α mRNA levels were higher than that in the arsenic group (Figure 5(a) to (e)). Similar effects were also observed with BAY 11–7082 (Figure 5(f) to (j)).

Effects of As_2O_3, GSPE, and BAY 11–7082 on the protein expression of p-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 in BEAS-2B cells

Arsenic increased the levels of p-IκB-α, IKKα, NF-κBp65, and NF-κBp50, while IκB-α levels were decreased significantly as compared with control (Figure 6(a) to (e)). Treatment with GSPE weakened the expression of p-IκB-α, IKKα, NF-κBp65, and NF-κBp50, and elevated IκB-α expression compared with the arsenic group (Figure 6(a) to (e)). In comparison with the arsenic group, BAY 11–7082 attenuated the levels of P-IκB-α, IKKα/β, NF-κBp65, and NF-κBp50, and increased IκB-α level (Figure 7(a) to (e)).

Effects of arsenic and GSPE on lung histology

Lung histology in mice showed no pathological changes in the lung tissue of the control and GSPE groups (Figure 8(a) and (b)). The alveolar septa of the arsenic group were obviously widened, with capillary congestion and inflammatory cell invasion (Figure 8(c)). In the presence of GSPE, compared with the arsenic group, the alveolar septa were narrowed, capillary congestion was decreased, and the number of inflammatory cell invasion was diminished (Figure 8(d)).

Effects of GSPE on inflammatory cytokines in lung tissue induced by arsenic in mice

Arsenic elevated the levels of IL-1β, IL-6, TNF-α, and CRP, and reduced IL-10 compared with controls. Compared with the arsenic group, IL-1β, IL-6, TNF-α, and CRP levels were decreased in the As_2O_3+GSPE group, while the IL-10 levels were increased (Figure 9(a) to (e)).

Effects of arsenic and GSPE on the mRNA expression of IκB-α, IKKα, IKKβ, NF-κBp65, and NF-κBp50 in the lung tissues of mice

Arsenic decreased the mRNA levels of IκB-α, while increased the mRNA levels of IKKα, IKKβ, NF-κBp65, and NF-κBp50 compared with controls. Compared with the arsenic group, the mRNA levels of IκB-α in As_2O_3 + GSPE group were elevated and the mRNA levels of IKKα, IKKβ, NF-κBp65, and NF-κBp50 attenuated (Figure 10(a) to (e)).

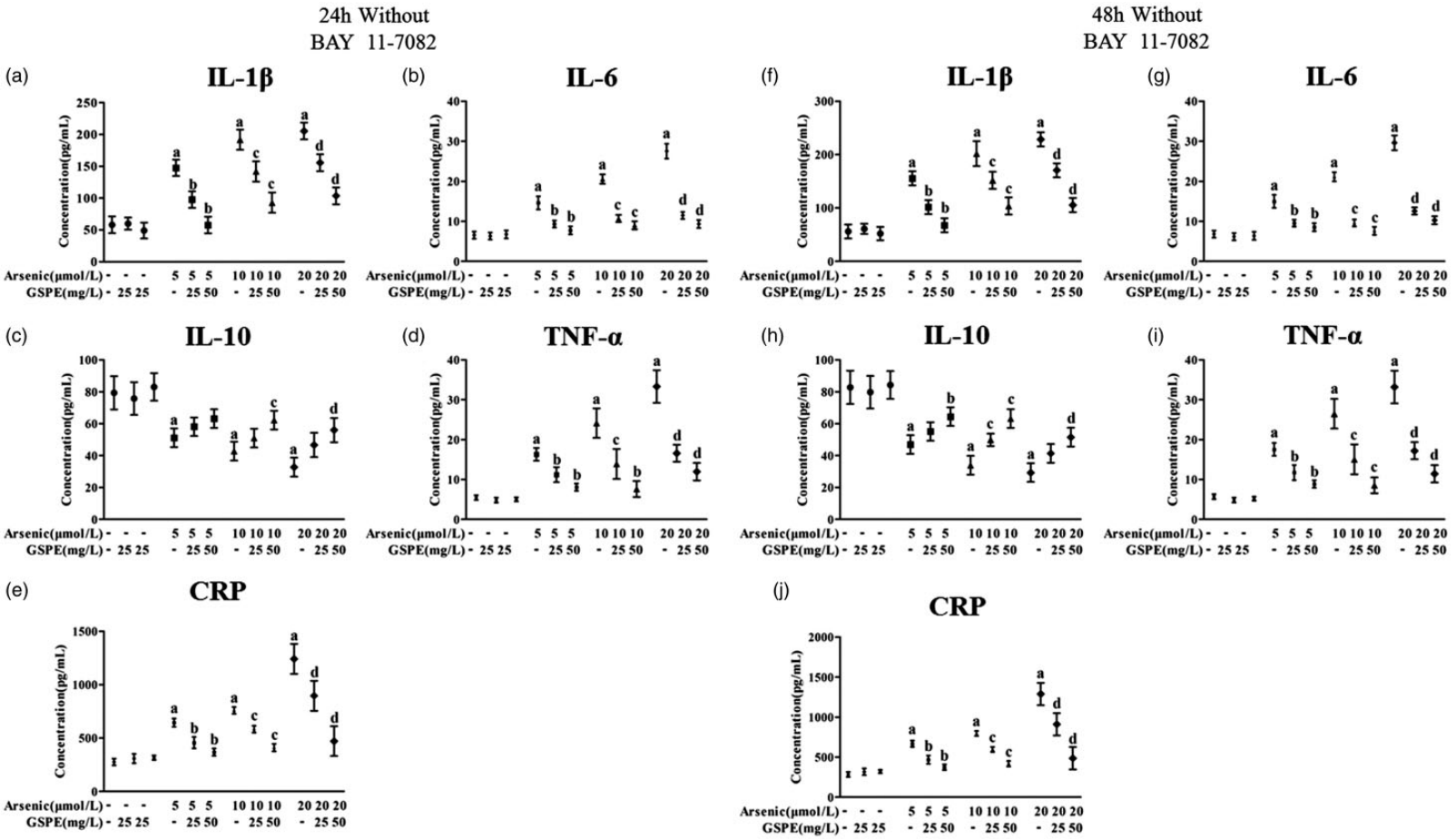

Figure 3. Effects of As_2O_3, GSPE on inflammatory cytokines of in BEAS-2B cells. The BEAS-2B cells were treated with As_2O_3 (0, 5, 10, or 20 μmol/L) (n = 3). And GSPE (0, 25, or 50 mg/L) (n = 3) was used to antagonize the effect of arsenic. After 24 h or 48 h, the cells were harvested to measure the expression of inflammatory cytokines. (a, f) IL-1β; (B, G) IL-6; (c, h) IL-10; (d, i) TNF-α; and (e, j) CRP. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group (5 μmol/L); $^{c}P < 0.05$, compared with the arsenic group (10 μmol/L); $^{d}P < 0.05$, compared with the arsenic group (20 μmol/L).
CRP: C-reactive protein; TNF-α: tumor necrosis factor-alpha; IL: interleukin; GSPE: grape seed proanthocyanidin extract.

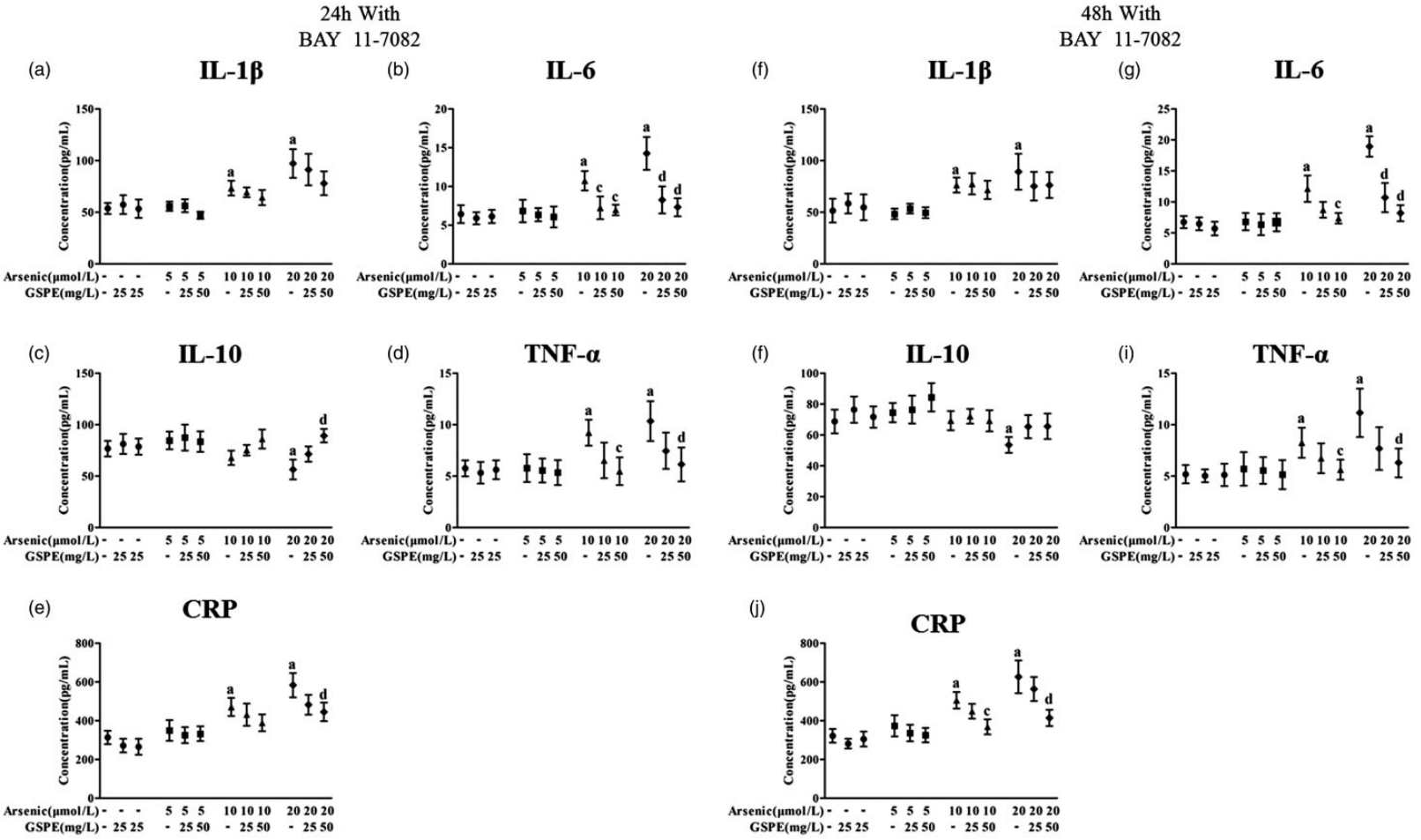

Figure 4. Effects of As_2O_3, GSPE, and BAY 11–7082 on inflammatory cytokines of in BEAS-2B cells. The BEAS-2B cells were treated with As_2O_3 (0, 5, 10, or 20 μmol/L), GSPE (0, 25, or 50 mg/L), and BAY 11–7082 (n = 3) was used to antagonize the effect of arsenic. After 24 h or 48h, the cells were harvested to measure the expression of inflammatory cytokines. (a, f) IL-1β, (b, g) IL-6, (c, h) IL-10, (d, i) TNF-α, and (e, j) CRP. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group (5 μmol/L); $^{c}P < 0.05$, compared with the arsenic group (10 μmol/L); $^{d}P < 0.05$, compared with the arsenic group (20 μmol/L).

CRP: C-reactive protein; TNF-α: tumor necrosis factor-alpha; IL: interleukin; GSPE: grape seed proanthocyanidin extract.

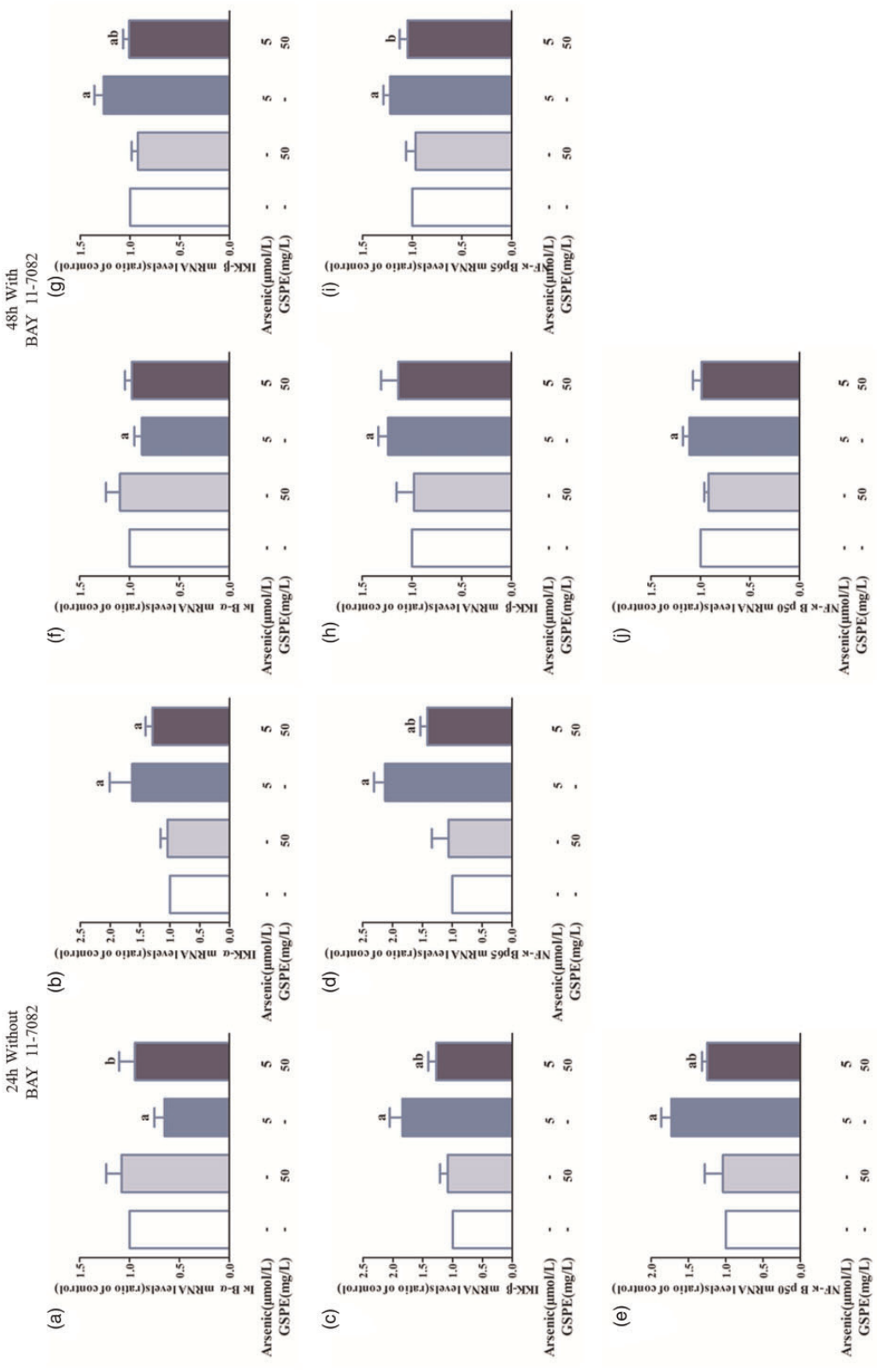

Figure 5. Effects of As_2O_3, GSPE, and BAY 11–7082 on the mRNA expression of IκB-α, IKKα, IKKβ, NF-κBp65, and NF-κBp50 in BEAS-2B cells. The BEAS-2B cells were treated with 5 μmol/L As_2O_3 (n = 10) and GSPE (50 mg/L) and BAY 11–7082 (50 mg/L) were used to antagonize the effects of arsenic. The relative mRNA levels were determined by RT-PCR using gene-specific primers. Values are expressed as means ± SD. $^aP < 0.05$, compared with the blank control group; $^bP < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

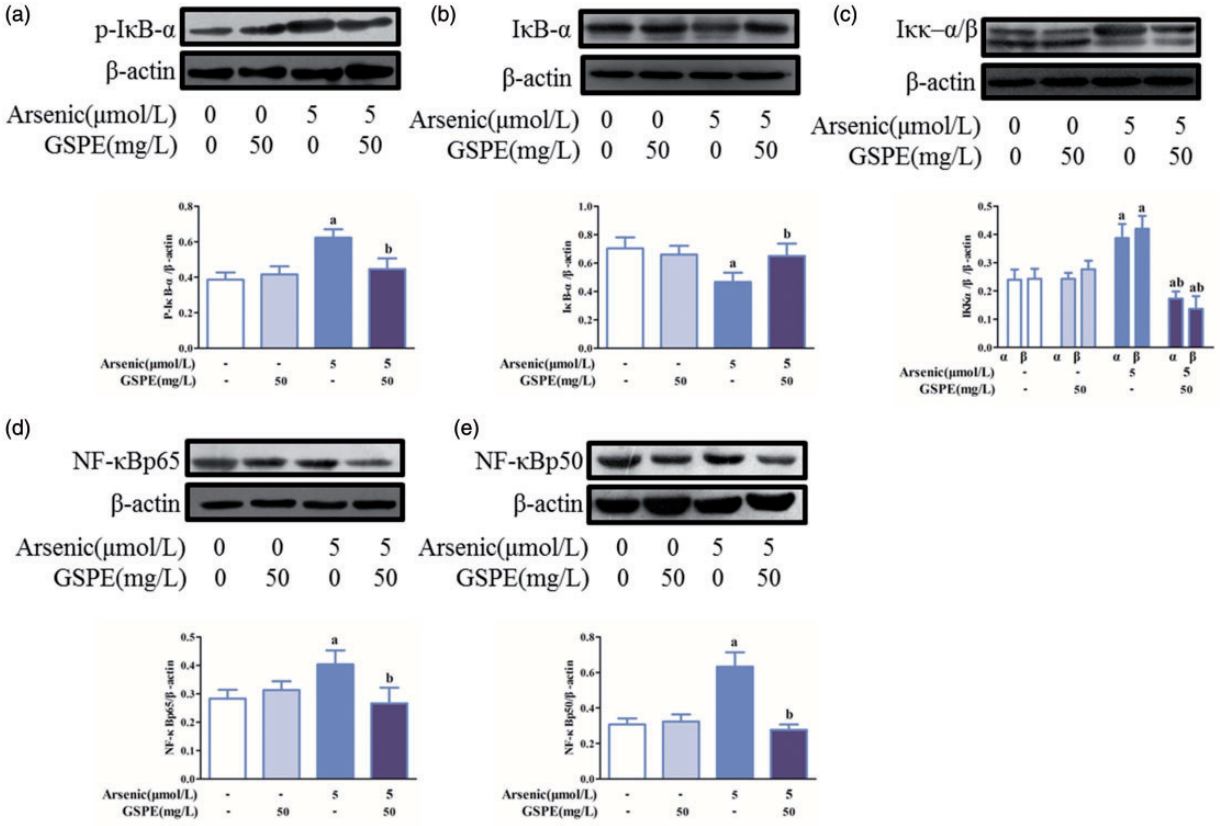

Figure 6. Effects of As_2O_3, GSPE on the protein expression of p-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 in BEAS-2B cells. The BEAS-2B cells were treated with 5 μmol/L As_2O_3 (n = 3). GSPE (50 mg/L) was used to antagonize the effect of arsenic. The expression of the various proteins was detected by Western blot. The levels of p-IκB-α (a), IκB-α (b), IKKα/β (c), NF-κBp65 (d), and NF-κBp50 (e) (without BAY 11–7082), respectively. The Image J software was used to measure the gray value of each blot and β-actin was used as a loading control. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

Effects of GSPE on the protein expression of P-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 in lung tissues induced by arsenic

Higher levels of P-IκB-α, IKKα/β, NF-κBp65, and NF-κBp50, and lower levels of IκB-α in the arsenic group were observed compared with the control group. Lower level of P-IκB-α, IKKα/β, NF-κBp65, and NF-κBp50, and higher level of IκB-α in the As_2O_3+GSPE group were observed compared with the arsenic group (Figure 11(a) to (e)).

Discussion

PC has beneficial features such as free radical scavenging, anti-inflammation, and anti-oxidation. There is no study on whether PC could protect against arsenic-induced respiratory inflammatory damage. Therefore, the aim of the present study was to examine the anti-inflammatory effects of PC and the molecular mechanisms in vivo and in vitro. The results suggest that arsenic induced damage in lung cells and mouse lungs. The NF-κB signaling pathway and inflammatory changes were involved in these effects. GSPE attenuated these changes in vitro and in vivo.

Arsenic is widely distributed in air, food, and water.[1,36] Arsenic exposure, through ingestion or contact, can cause toxic damage to multiple organs.[37] Arsenic can promote the expression of IL-6, IL-8, and TNF-α, and reduce IL-10 expression, thereby resulting in inflammation and decreased cell viability.[4] In the present study, in vitro, arsenic induced cell damage, since decreased cell viability and increased cell apoptosis were observed when BEAS-2B cells were exposed to arsenic. This damage could be alleviated by GSPE or BAY 11–7082 treatment, as previously observed.[35] Arsenic activated the NF-κB signaling pathway by increasing the mRNA levels of IKKα, IKKβ, NF-κBp65, and NF-κBp50, and protein levels of P-IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 as well as by decreasing the mRNA and protein expression of IκB-α. These changes led to the altered expression of downstream factors and inflammatory damage of BEAS-2B cells following upregulation of the expression of IL-1β, IL-6, TNF-α, CRP, ROS, and LPO, and downregulation of IL-10. Nevertheless, this arsenic-induced inflammation seemed to be antagonized by GSPE since it decreases the levels of IL-1β, IL-6, TNF-α, CRP, ROS, and LPO, and increases the IL-10 levels, which could result from the suppression of the NF-κB signaling. These effects of arsenic and GSPE were also observed in vivo. A previous study showed that GSPE (400 mg/kg) by intragastric administration, for five weeks, did not alter body weight, lung weight, and viscera coefficient of normal Kunming mice. On the other hand, decreases in body weight and increases in lung weight and viscera coefficient were

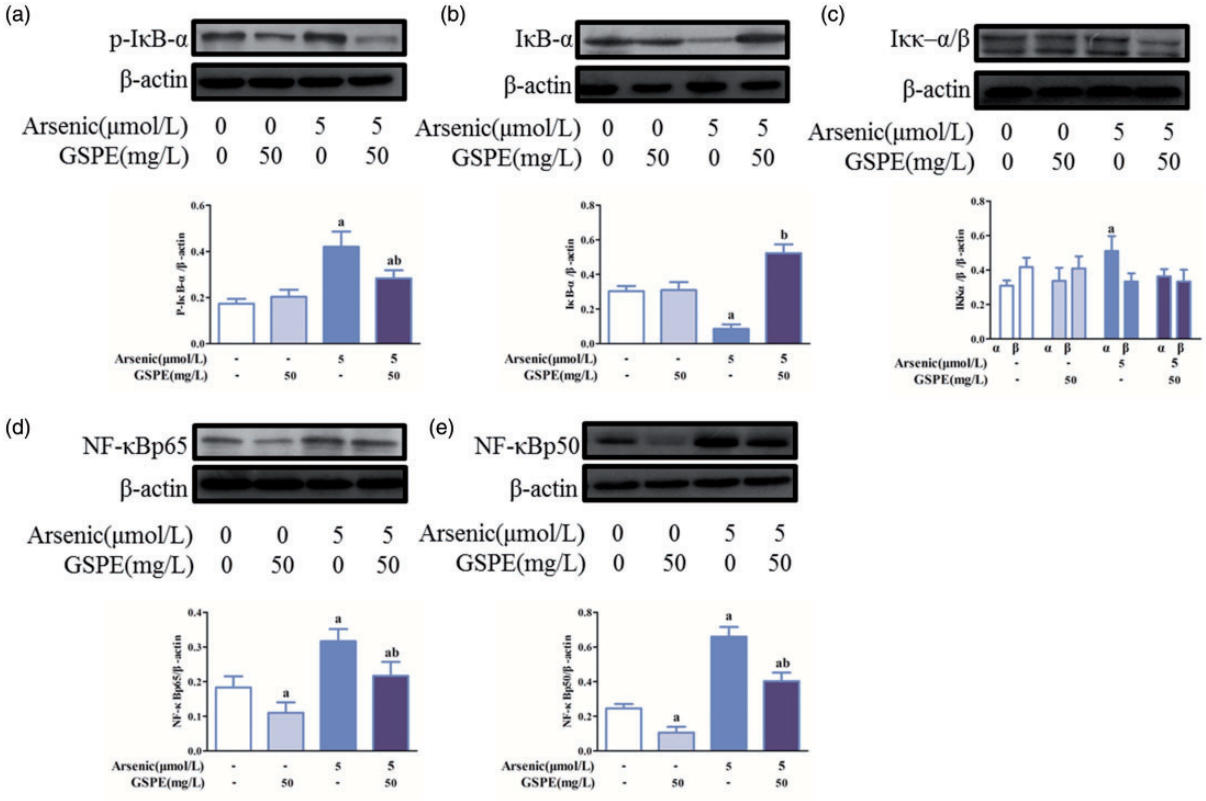

Figure 7. Effects of As_2O_3, GSPE, and BAY 11–7082 on the protein expression of p-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 in BEAS-2B cells. The BEAS-2B cells were treated with 5 μmol/L As_2O_3 (n = 3). GSPE (50 mg/L) was used to antagonize the effect of arsenic. The expression of the various proteins was detected by Western blot. The levels of p-IκB-α (a), IκB-α (b), IKKα/β (c), NF-κBp65 (d), and NF-κBp50 (e) (with BAY 11–7082), respectively. The Image J software was used to measure the gray value of each blot and β-actin was used as a loading control. Values are expressed as means ± SD. $^aP < 0.05$, compared with the blank control group; $^bP < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

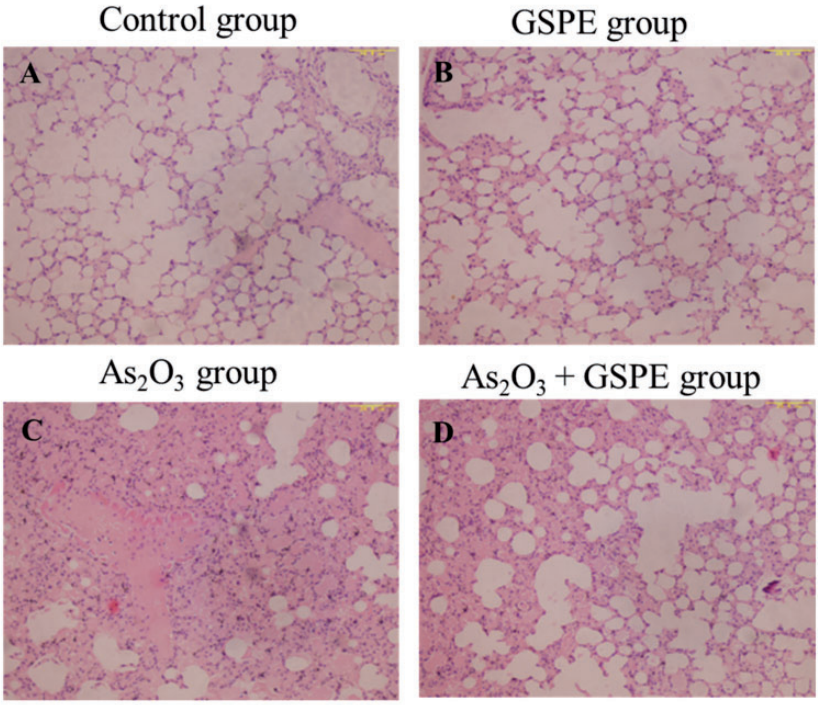

Figure 8. Representative photomicrographs of H&E stained section of lung after treatment with arsenic and GSPE. (a) Control group, (b) GSPE group, (c) As_2O_3 group, (d) As_2O_3+GSPE group (×200). (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

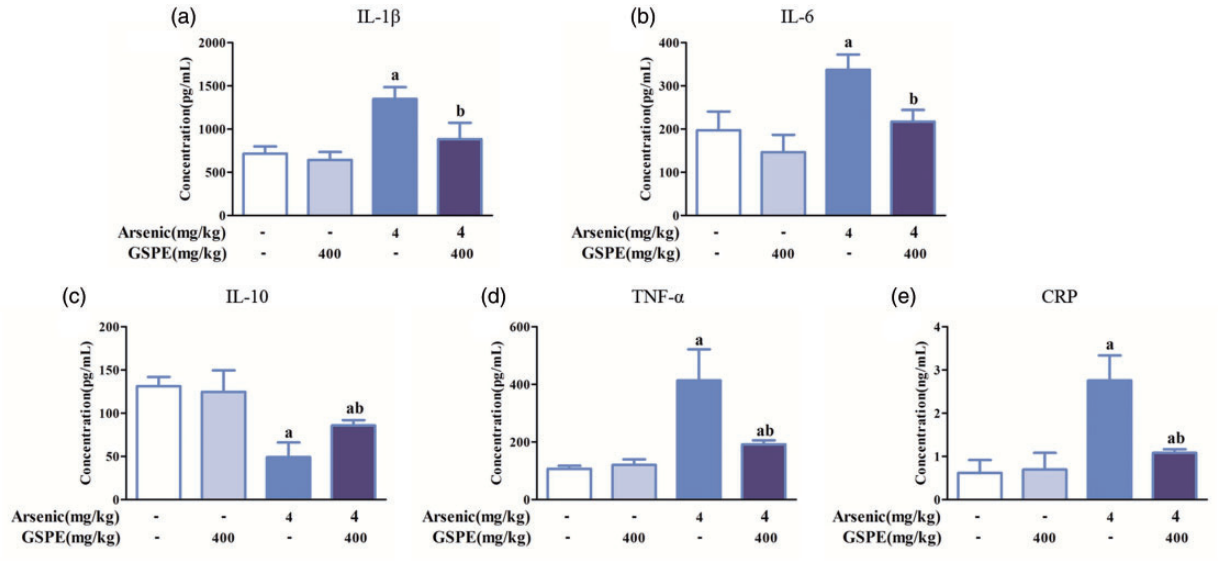

Figure 9. Effects of arsenic and GSPE on inflammatory cytokines expression in lung tissue induced by arsenic in mice. Mice were exposed to As_2O_3 (4 mg/kg) (n = 10); 400 mg/kg GSPE was used to antagonize the effects of arsenic. Levels of IL-1β (a), IL-6 (b), IL-10 (c), TNF-α (d), and CRP (e), respectively. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

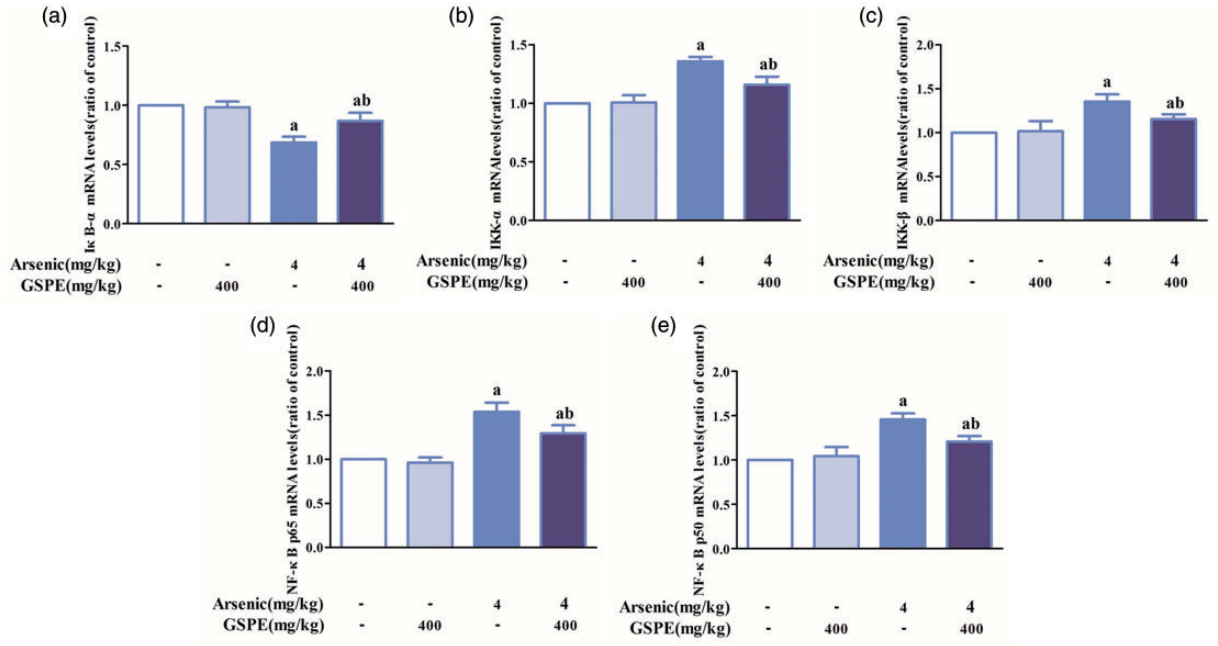

Figure 10. Effects of arsenic and GSPE on mRNA expression of IκB-α, IKKα, IKKβ, NF-κBp65, and NF-κBp50 in lung tissue induced by arsenic in mice. Mice were exposed to As_2O_3 (4 mg/kg) (n = 10); 400 mg/kg GSPE was used to antagonize the effects of arsenic. The expression of each mRNA in the control, GSPE, AsO_3, and As_2O_3+GSPE groups was detected by RT-PCR. mRNA levels of IκB-α (a), IKKα (b), IKKβ (c), NF-κBp65 (d), and NF-κBp50 (E), respectively. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

found after five weeks of intragastric administration of As_2O_3 (4 mg/kg) in normal mice. GSPE in arsenic-exposed mice for five weeks reduced the lung weight and viscera coefficient.[35] Taken together, these results strongly suggest that arsenic activates NF-κB signaling and promotes the expression of downstream inflammatory cytokines, thereby causing inflammatory damage. These effects could be blocked, at least in part, by GSPE.

It has been reported that the activation of NF-κB,[38] MAPK,[33] Nrf2,[39] and other signaling pathways may account for the arsenic-induced inflammatory damage. It is believed that NF-κB signaling pathway plays a

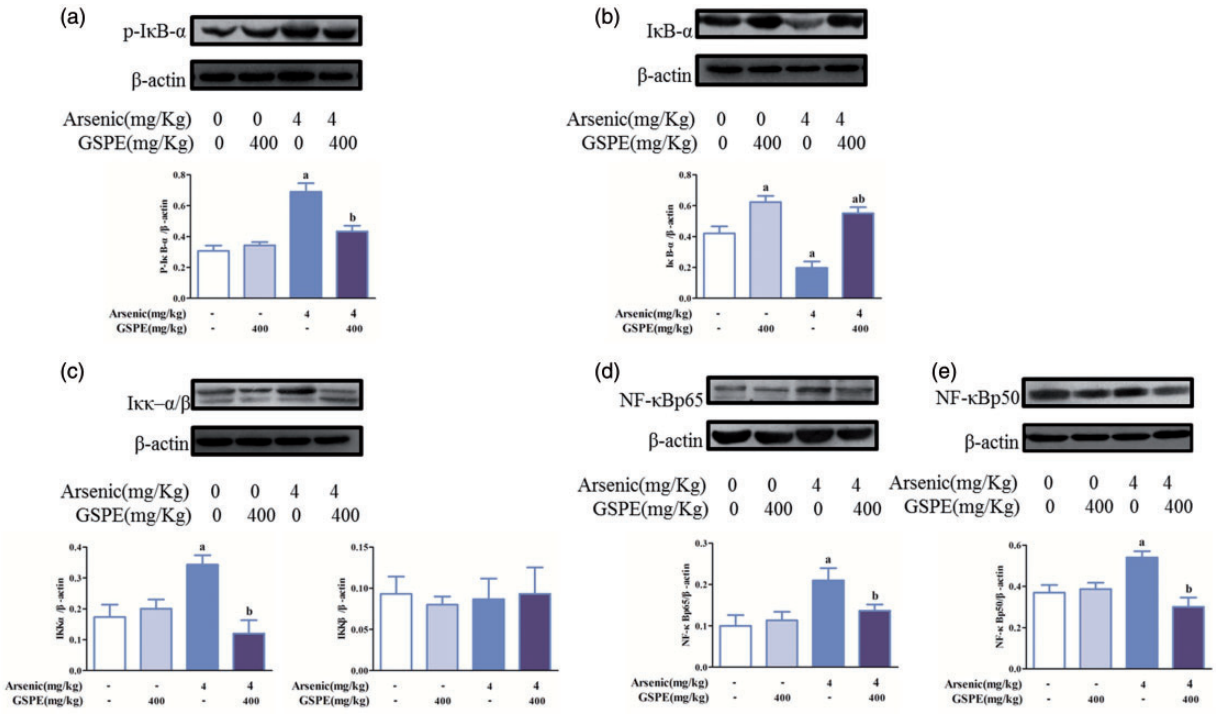

Figure 11. Effects of GSPE on the protein expression of p-IκB-α, IκB-α, IKKα/β, NF-κBp65, and NF-κBp50 in lung tissues induced by arsenic. Mice were exposed to As_2O_3 (4 mg/kg) (n = 10); 400 mg/kg GSPE was used to antagonize the effects of arsenic. The expression of each protein in the control, GSPE, AsO_3, and As_2O_3+GSPE groups was detected by Western blot. Protein levels of p-IκB-α (a), IκB-α (b), IKKα/β (c), NF-κBp65 (d), and NF-κBp50 (E), respectively. The Image J software was used to measure the gray value of each blot and β-actin was used as a loading control. Values are expressed as means ± SD. $^{a}P < 0.05$, compared with the blank control group; $^{b}P < 0.05$, compared with the arsenic group. (A color version of this figure is available in the online journal.)
GSPE: grape seed proanthocyanidin extract.

prominent role in inflammatory damage, as suggested by the present study. One of the possible mechanisms for these effects was that arsenic promoted the phosphorylation of IκB-α, enhanced the ubiquitination and degradation of IκB-α,[40] accelerated the dissociation of the complex of IκB-α, NF-κBp65, and NF-κBp50, released the p50-p65 dimers,[41] and then allowed the dimers to become free to translocate to the nucleus from the cytoplasm, to combine with the DNA binding site of κB in order to activate the NF-κB signaling pathway and the expression of inflammatory cytokines.[42] It is also likely that arsenic increased the activity and expression of IKK and facilitated the dissociation of the complex, thereby activating the NF-κB signaling pathway.[42] In addition, arsenic has been shown to boost the DNA-binding activity of NF-κBp65 and NF-κBp50 that had entered the nucleus.[42] It had been reported that arsenic could enhance the expression of INF-γ, TNF-α, IL-6, VEGF, HO-1, ROS, and LPO[43,44] and decrease the levels of IL-10 and IL-4.[45] A previous study showed that when the BEAS-2B cells were treated with As_2O_3 for 24 h and 48 h, the levels of ROS and LPO were significantly increased in a dose-dependent manner.[35] In contrast, the expression of ROS and LPO were decreased with the increasing GSPE concentration. A decreasing trend in the present study was shown in ROS and LPO when BAY 11–7082 was applied. A previous study showed that the levels of LPO and ROS in the GSPE group were similar with the control group. LPO and ROS were elevated when mice were exposed to As_2O_3 (4 mg/kg) and attenuated by GSPE.[35] In the present study, arsenic caused inflammatory damage with elevated levels of IL-1β, IL-6, and TNF-α, and loss of IL-10 expression. An involved mechanism may be that IL-6 promoted the expression of CRP[44] and the elevated TNF-α levels enhanced the content of ROS and LPO.[46] Meanwhile, the secreted IL-1β could bind to its receptor on the cell membrane and further act on IKK, aggravating the degree of inflammatory damage.[47]

BAY 11–7082 is a known inhibitor of the NF-κB signaling pathway and can effectively inhibit the phosphorylation and degradation of IκB-α. In the present study, we investigated whether GSPE, which had been shown to have good effect on anti-inflammatory and scavenging free radicals,[21] could diminish the arsenic-induced inflammatory damage by inhibiting NF-κB signaling. We found that GSPE restrained the NF-κB signaling pathway and alleviated the inflammatory damage caused by arsenic, as expected, in that GSPE decreased IKK expression, inhibited IκB-α phosphorylation, and blocked the formation of NF-κBp65 and NF-κBp50, thereby reducing the expression of IL-1β, IL-6, TNF-α, and CRP, and promoting IL-10 expression. Arsenic-induced inflammatory damage (alveolar morphology changes, lung congestion, and inflammatory cells invasion) was prevented by GSPE to a certain extent. The anti-inflammatory effect of GSPE had also been reported in other studies,[20,35] and the beneficial effects of GSPE could be derived from the inhibition of the NF-κB signaling pathway.[19] GSPE administration could antagonize the effects of arsenic by inhibiting arsenic-induced NF-κB

activation, as supported by those previous studies. Supplementary Figure 1 summarizes the effects of arsenic and GSPE, as observed in the present study.

The present study found antagonistic effects of GSPE on NF-κB signaling pathway and inflammatory damage induced by arsenic. Nevertheless, PI3K and Akt have important regulative effects on NF-κB signaling.[48] MAPK signaling pathways were also reported to modulate the damage induced by arsenic.[49] In addition, arsenic-induced inflammatory damage might also be related to ERK, Stat3, and other signaling pathways.[50] Future studies should explore the molecular mechanisms of multiple signaling pathways involved in inflammation and interactions with arsenic and GSPE. Future studies will also have to include a positive control, which was a limitation of the present study. The pathways involved in arsenic-induced damage and rescue by GSPE will also have to be studied using a variety of inhibitors and siRNAs.

Arsenic-activated NF-κB signaling induced inflammatory damage through the upregulation of pro-inflammatory cytokines and downregulation of anti-inflammatory cytokines. GSPE plays a beneficial role against arsenic-induced inflammatory damage through, at least in part, the suppression of the arsenic-induced NF-κB signaling pathway. Therefore, GSPE may represent a potential agent to prevent inflammatory damage induced by arsenic exposure.

Authors' contributions: YHH, MW contributed equally to this work. YHH, MW, SGL and LJP carried out the studies, participated in collecting data, and drafted the manuscript. QN, RLM, YL, XHW and GLF performed the statistical analysis and participated in its design. SGL and LJP helped to draft the manuscript. All authors read and approved the final version of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

This study was supported by the National Natural Science Foundation of China (Nos. 81760584, 81560517); the Key Areas of Science and Technology Research Project of Xinjiang Production and Construction Corps (No. 2014BA039 and No. 2015AG014); and the International Cooperative Project of Shihezi University (No. GJHZ201602).

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(Received September 13, 2018, Accepted January 18, 2019)