Protective Effects of Gallic Acid Against NiSO$_4$-Induced Toxicity Through Down-Regulation of the Ras/ERK Signaling Pathway in Beas-2B Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

BCDEF Xuejun An
BCDF Aijia Zhou
BCDE Yue Yang
BCD Yue Wang
BCD Rui Xin
BCD Chao Tian
ADG Yonghui Wu

Department of Occupational and Environmental Health, School of Public Health, Harbin Medical University, Harbin, Heilongjiang, P.R. China

Corresponding Author: Yonghui Wu, e-mail: wuyonghui777@163.com
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Background: This study aimed to explore the preventive effects of gallic acid (GA) on the toxicity induced by NiSO$_4$ in Beas-2B cells.

Material/Methods: Beas-2B cell viability was measured by MTT assay. The degree of oxidative stress was detected by measuring the levels of reactive oxygen species (ROS) and lipid peroxide (LPO). The rate of apoptosis was measured by flow cytometry. Ras/ERK-related protein levels were analyzed by Western blot analysis, which including Ras, ERK, c-Myc, PARP, and PARP cleavage.

Results: MTT assay showed that NiSO$_4$ induced cytotoxicity, while GA had a protective role against toxicity. Additionally, GA could reduce the apoptotic cell number and the level of ROS in Beas-2B cells induced by NiSO$_4$. Western blot analysis demonstrated that NiSO$_4$ could up-regulate the related protein in the Ras/ERK signaling pathway. Furthermore, we observed that GA could alleviate the toxicity of NiSO$_4$ through regulating protein changes in the Ras/ERK signaling pathway.

Conclusions: Preventive effects of GA on NiSO$_4$-induced cytotoxicity in Beas-2B cells may be through the Ras/ERK signaling pathways.

MeSH Keywords: Gallic Acid • Nickel • Preventive Medicine

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Background

Nickel compounds are well-known carcinogens that have been identified in industrial environments, such as plating, aluminum coloring, battery production, catalyst, etc. Nickel compounds can enter the body through inhalation, ingestion, and dermal absorption, and inhalation is a main route to cause respiratory disease [1]. Both *in vitro* and *in vivo* experiments have demonstrated that respiratory distress and lung and nasal cancer are the common adverse health effects of exposure to nickel compounds [2–4]. A large body of evidence indicates that soluble nickel compounds such as nickel sulfate ($NiSO_4$) and nickel chloride ($NiCl_2$) may cause the cellular production of reactive oxygen species (ROS) and lipid peroxide (LPO) [5–7]. Therefore, the potential harm of nickel compounds for occupational workers cannot be neglected. However, the molecular mechanism and critical signaling pathways by which nickel compounds induce cytotoxicity, oxidative damage, and apoptosis still remain elusive.

Gallic acid (3,4,5-trihydroxy benzoic acid, GA) is a kind of polyphenol compound in nature with high medicinal value, which widely exists in grapes, pomegranates, black tea, and traditional Chinese medicine, such as Moutan, dogwood, gallnut, saxifrage, etc. [8,9]. GA is known to have multiple pharmacological functions, such as anti-carcinogenic, anti-hepatitis B virus, anti-HIV, trypanocidal activity, anti-inflammatory, antibacterial, and antiviral [10–12]. However, the potential protective effect of GA against nickel compounds' toxicity has not been investigated. This study was conducted to explore cytotoxicity, oxidative stress, and apoptosis induced by $NiSO_4$ *in vitro* culture of human bronchial epithelial Beas-2B cells. The potential preventive effect of GA against $NiSO_4$-induced toxicity was further researched. In the present study, we chose human bronchial epithelial Beas-2B cells. Since the bronchial epithelium is an important protective barrier against inhalable particle matter, and inhalation is the main way for humans to be exposed to nickel compounds, the Beas-2B cell line is a suitable model to research the toxic effect of nickel compounds.

The Ras/ERK signaling pathway family includes Ras, MEK, ERK, c-Myc, etc. c-Myc is a member of the Myc family of transcription factors, and it is frequently overexpressed in abnormal cells [13]. By modifying the expression of target gene, c-Myc leads to a variety of biological effects, including regulation of cell growth, promotion of cell proliferation, apoptosis, stem cell self-renewal, and DNA damage response [14]. PARP is a chromatin-associated enzyme that is very important for the stability and survival of cells. PARP cleavage is thought to be an important marker of apoptosis, and it is also generally considered to be a marker of caspase-3 activation [3]. Therefore, we chose Ras, ERK, c-Myc, PARP, and PARP cleavage in the Ras/ERK pathway to carry out the experiment.

As far as we know, this study is the first to report the potential protective effect of GA against $NiSO_4$-induced cell injury through the Ras/ERK signaling pathway.

Material and Methods

Reagents and antibodies

Nickel sulfate ($NiSO_4$) (Product No.: 851028, purity≥98%) was obtained from Qingong Chemical Plant in Shanghai, China, and gallic acid (GA) (Lot No.: M0116A, purity >98%) was bought from Meilun Biological Company in Dalian, China. The reagent kits for ROS, lactate dehydrogenase (LDH), malondialdehyde (MDA), and glutathione (GSH) were purchased from Jiancheng Biosciences, China. Annexin V-FITC Apoptosis Detection Kit I was purchased from BD Biosciences, USA. The following antibodies were used: Ras, phosphor-ERK1/2, ERK1/2, phosphor-c-Myc, c-Myc, PARP, PARP cleavage antibody, β-actin antibody, and secondary antibody (Cell Signaling Technology, USA). Manumycin A (Ras inhibitor) was from Abcam, USA, and PD98059 (ERK inhibitor) was from Tocris Bioscience, UK. PhosSTOP was from Roche Bioscience, Germany. All other reagents were purchased from Sigma Chemical Company in the USA unless otherwise specified.

Cell culture conditions

Human bronchial epithelial Beas-2B cells were obtained from the Cell Bank of the Chinese Academy of Sciences. Beas-2B cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning). The culture medium contained 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin. Cells were maintained at 37°C as monolayers in a humidified atmosphere containing 5% CO_2.

MTT assay

The viability of Beas-2B cells was measured by MTT assay as described by Ahamed et al. with some specific modification [15].

Cellular reactive oxygen species content determination

The determination of cellular ROS content was strictly carried out according to the kit instructions. In summary, Beas-2B cells were seeded into 24-well cell culture plates at a concentration of $5×10^4$ cells/well. After treatment, cells were collected and incubated with 1 mL of DCFH-DA at 37°C for 20 min. In addition, this experiment needed to set a positive control. The cells were washed 3 times with DMEM and observed under laser scanning confocal microscope at once (Leica TCS SP5, Germany). The chemical fluorescence method was used to detect the activity of ROS in different concentrations of GA for 24 h.

Cellular glutathione, lactate dehydrogenase, and malondialdehyde content determination
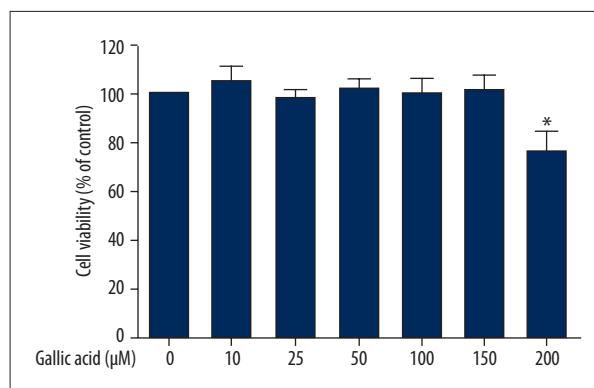
The determination of cellular LDH, MDA, and GSH content was strictly carried out according to the kit instructions. After treatment, cells were evaluated by Microplate Reader (Thermo Fisher Scientific, USA).

Flow cytometry to examine the apoptosis

The apoptosis rate was detected by Annexin V-FITC Apoptosis Detection Kit I. In summary, after treatment, Beas-2B cells were washed twice with pre-cooling PBS and added to a flow cytometry tube with 500 µL of Annexin binding buffer; then 5 µL of Annexin-V-FITC and 5 µL of PI were added to each tube. Finally, cells were gently mixed with avoidance of light at room temperature for 15 min. The apoptosis rate was examined using FACSCalibur (BD Biosciences, USA).

Western blot analysis

The extraction method for total protein and Western blot were introduced in a previous study. In summary, after treatment, cells were washed twice with PBS and split in a cell lysis buffer containing 1 mM PMSF and 1 mM PhosSTOP on ice for 50 min. Then, the cell suspension was centrifuged for 10 min at 12,000 rpm at 4°C, and supernatant was collected. The total protein concentration was determined by using the BCA Protein Assay

kit. Protein (20 µg-40 µg) of each group lysate was separated using 8–10% (w/v) SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk in tris-buffered saline with Tween 20 (TBST) for 30 min at 37°C, and incubated with rabbit-polyclonal primary antibodies against RAS (1: 1000), p-ERK1/2 (1: 1000), ERK1/2 (1: 1000), p-c-Myc (1: 1000), c-Myc (1: 1000), PARP (1: 1000), PARP cleavage (1: 1000), and β-actin (1: 1000)


Figure 1. Effect of GA on cell viability. Beas-2B cells were exposed to different concentrations of GA for 24 h, and cell viability was determined by MTT assay as described in the Material and Methods section. Data represent mean ±SD of three independent experiments made in three replicates. * *P*<0.05, compared with control group.
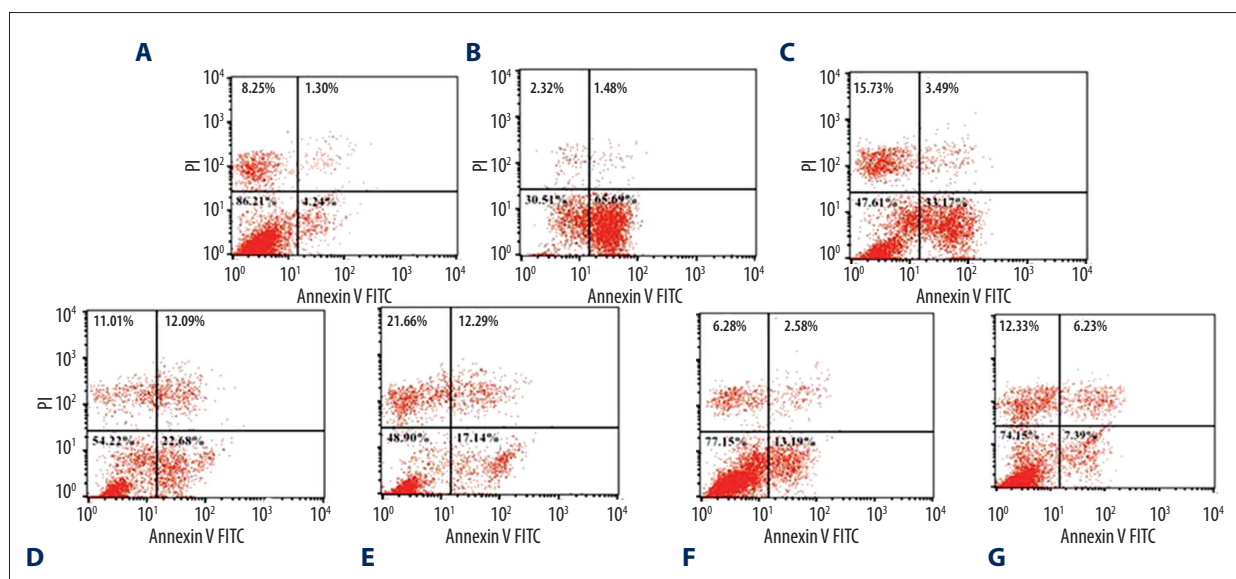
Figure 2. Effect of GA on NiSO₄-induced apoptosis. Beas-2B cells were exposed to 500 µM NiSO₄ for 24 h in the presence or advance of GA (0–150 µM), and apoptosis was determined by flow cytometry analysis as described in the Material and Methods section. (**A**) Control group. (**B**) 500 µM NiSO₄. (**C**) 10 µM GA + 500 µM NiSO₄. (**D**) 25 µM GA + 500 µM NiSO₄. (**E**) 50 µM GA + 500 µM NiSO₄. (**F**) 100 µM GA + 500 µM NiSO₄. (**G**) 150 µM GA + 500 µM NiSO₄. Data represent mean ±SD of three independent experiments made in three replicates. * Significant preventive effect of different concentrations of GA on reduction of oxidative stress caused by NiSO₄ (*P*<0.05).
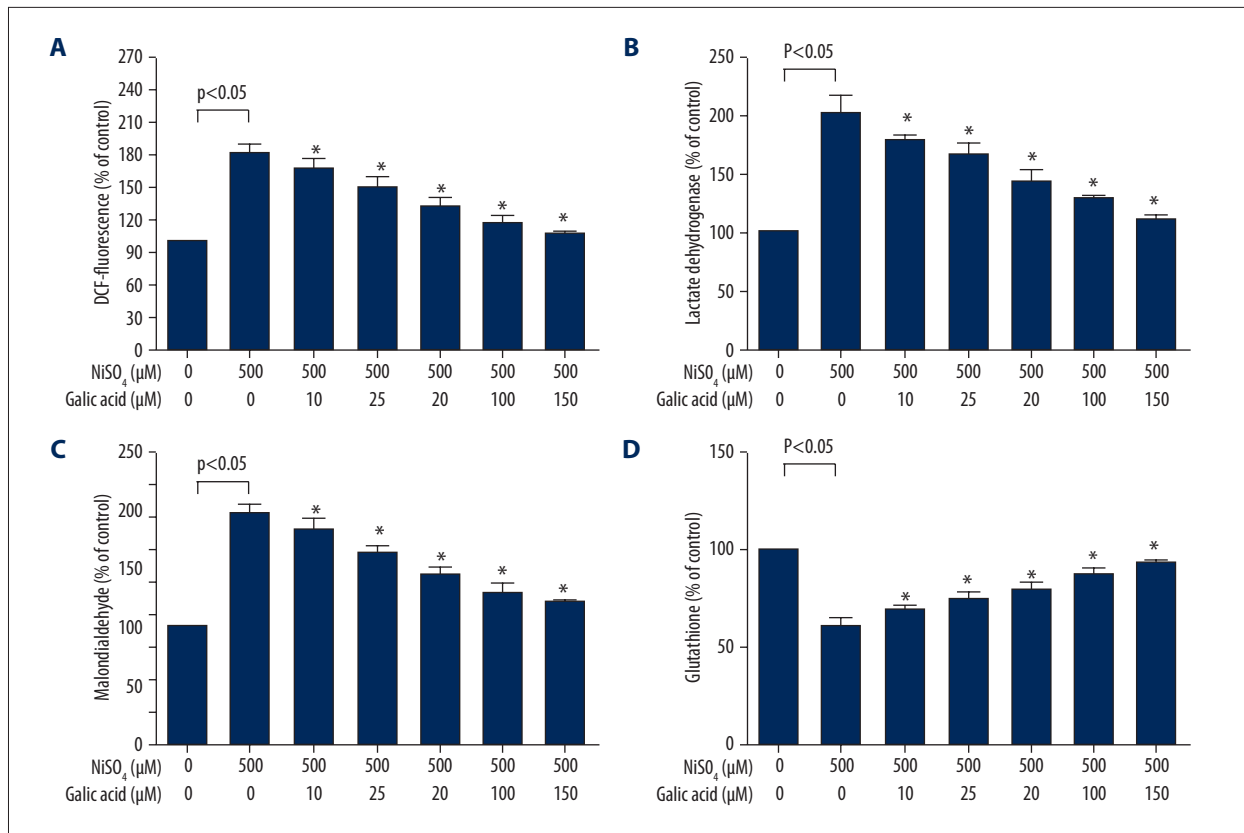
Figure 3. Effect of GA on NiSO$_4$-induced oxidative stress. Beas-2B cells were exposed to 500 µM NiSO$_4$ for 24 h in the presence or advance of GA (0–150 µM), and the cells' oxidative stress parameters were determined according to the kit instructions as described in the Material and Methods section. (**A**) ROS, (**B**) LDH, (**C**) MDA, and (**D**) GSH. Data represent mean ±SD of three independent experiments made in three replicates. * Significant preventive effect of different concentrations of GA on reduction of oxidative stress caused by NiSO$_4$ ($P<0.05$).

overnight at 4°C. In the next step, the blots were washed three times with TBST, followed by 30 min of incubation with appropriate goat anti-rabbit alkaline phosphatase (AP) conjugated secondary antibody (1: 2000). The antibody-reactive bands were monitored by stabilized substrate for alkaline phosphatase and its densitometry was quantified using ChemiAnalysis image analysis software (Clinx Science Instruments, China).

Statistical analysis

Each experiment was performed at least three times, and representative data are shown. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, and all statistical analysis was carried out using GraphPad Prism Software. $P<0.05$ was considered to be significant.

Results

Effect of GA on cell viability

Figure 1 shows the cell viability of Beas-2B cells exposed to GA (10 µM-200 µM) for 24 h. Results suggested that GA in the concentration range of 10 µM-150 µM did not decrease significant numbers of living cells ($P>0.05$). A higher concentration of GA (200 µM) induced 23.58% reduction in cell viability ($P<0.05$). The safe doses of GA (10–150 µM) were further used to research its protective effect.

Effect of GA on NiSO$_4$-induced apoptosis

The potential of GA to prevent the apoptosis induced by NiSO$_4$ in Beas-2B cells was examined. Cells were exposed to NiSO$_4$ at the concentration of 500 µM for 24 h in the presence of GA (10–150 µM). As shown in Figure 2, flow cytometry analysis indicated that co-exposure to GA (10–150 µM) had a significant preventive effect on apoptosis induced by NiSO$_4$.
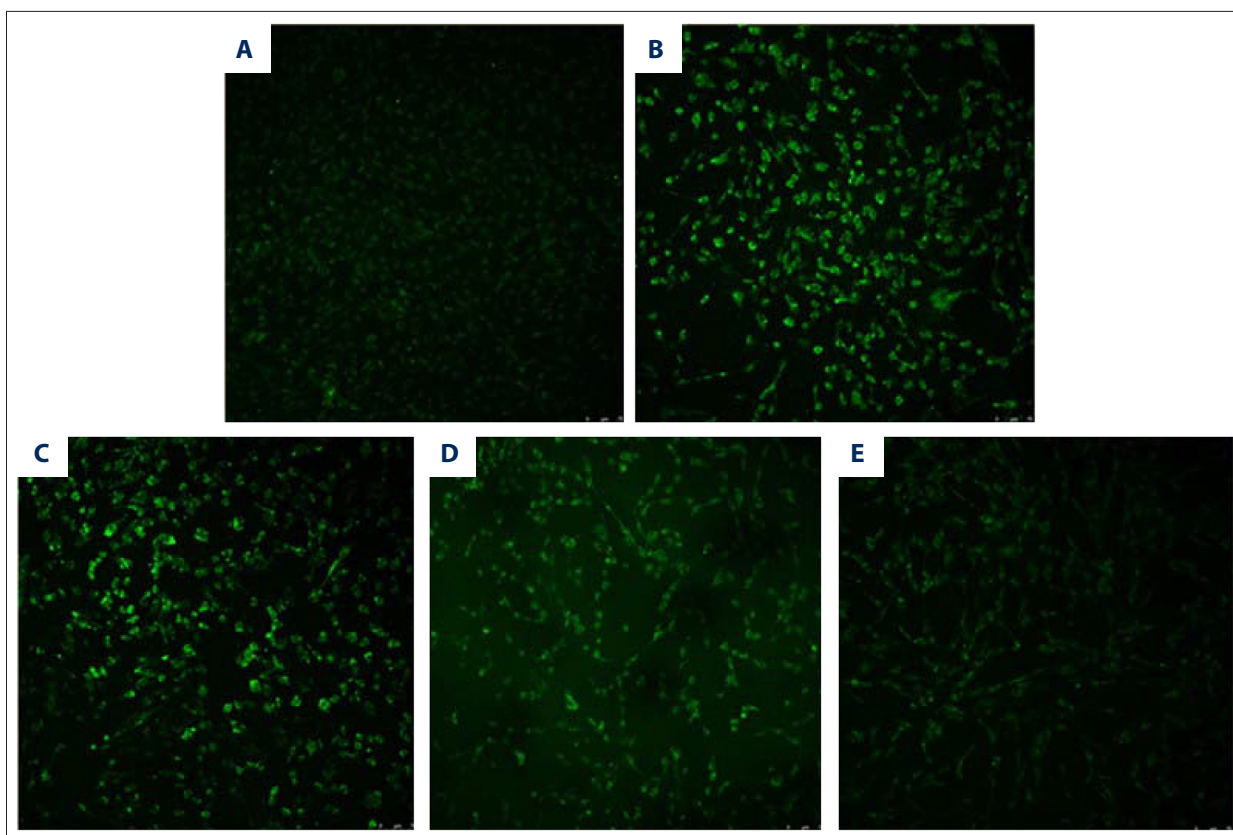
Figure 4. Effect of GA on NiSO$_4$-induced reactive oxygen species fluorescence intensity. The expression of reactive oxygen species fluorescence intensity was observed by laser confocal microscopy. (**A**) Control group. (**B**) 500 µM NiSO$_4$. (**C**) 10 µM GA+500 µM NiSO$_4$. (**D**) 50 µM GA+500 µM NiSO$_4$. (**E**) 150 µM GA+500 µM NiSO$_4$. The original magnification is 20×.

Effect of GA on NiSO$_4$-induced oxidative stress

To investigate the protective effect of GA on NiSO$_4$-induced oxidative stress, Beas-2B cells were first treated with GA (10 µM-150 µM) for 24 h and then incubated with 500 µM NiSO$_4$ for 24 h. We observed that co-exposure to GA significantly inhibited the oxidant (ROS, LDH, and MDA) induction and antioxidant (GSH) reduction in Beas-2B cells exposed to NiSO$_4$ ($P<0.05$) (Figure 3A–3D). The fluorescence intensity of Beas-2B cells treated with GA (10 µM, 50 µM, and 150 µM) for 24 h before NiSO$_4$ infection decreased compared with the only 500 µM NiSO$_4$-infected group (Figure 4).

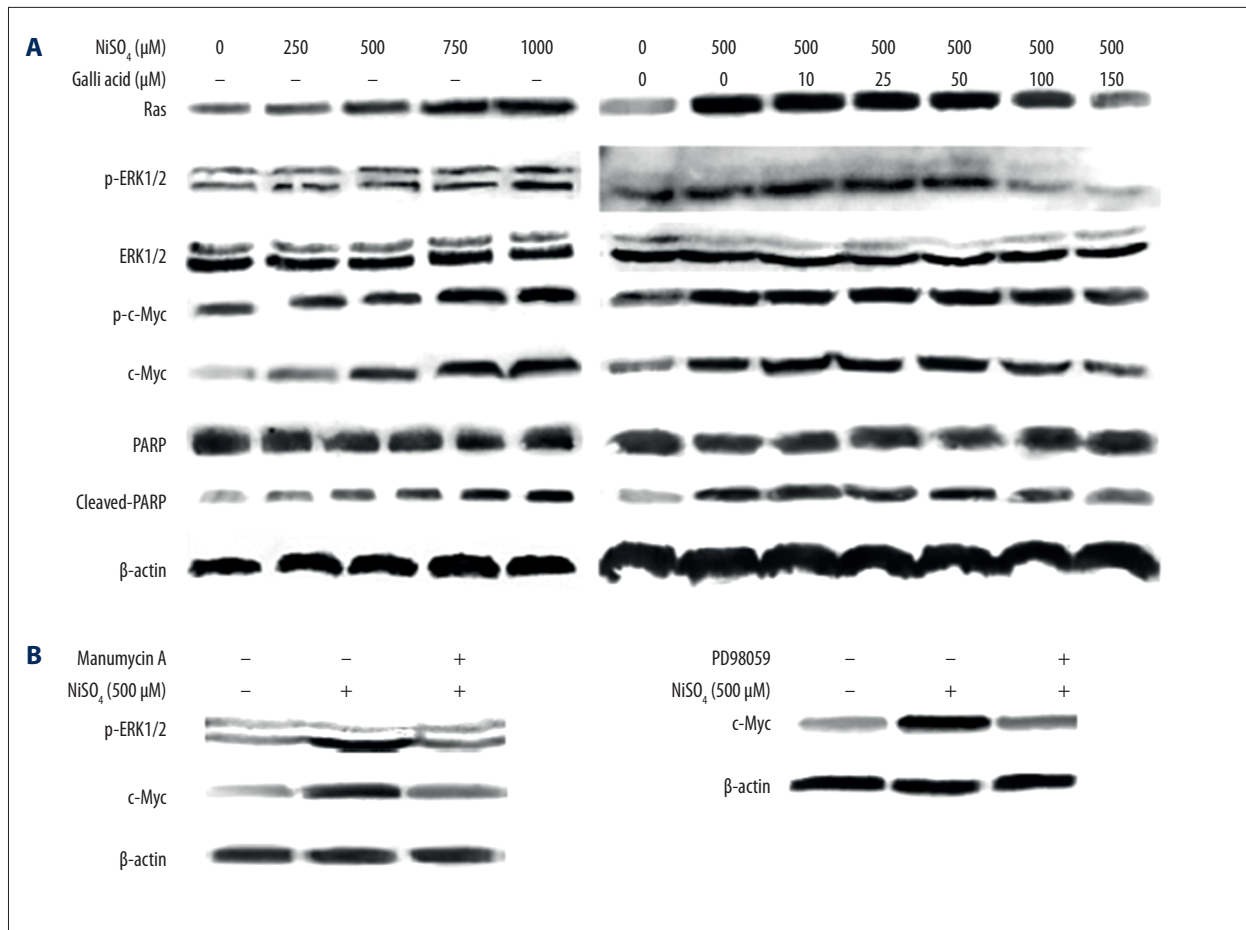### Effect of GA on NiSO$_4$-induced Ras/ERK signaling pathway protein expressions

Western blot analysis showed that the Ras protein level was increased by NiSO4 in Beas-2B cells after 24 h of exposure. Further, along with the concentration of Ras increasing, the levels of p-ERK1/2, c-Myc, and PARP cleavage also increased while PARP showed the opposite expression. We also observed that PARP cleavage, a marker of cell apoptosis, was increased by NiSO$_4$ (Figure 5A). Additionally, to research whether the

increased c-Myc protein was regulated by the Ras/ERK pathway, cells were treated with certain inhibitors for 1 h prior to the time that cells were exposed to NiSO$_4$ (500 µM) for 24 h. The results showed that both Ras and ERK inhibitors decreased NiSO$_4$-induced c-Myc protein (Figure 5B).

GA's depressive effect on NiSO$_4$-induced Ras, p-ERK1/2, c-Myc, and PARP cleavage protein up-regulation was observed in the groups co-exposed to GA (10 µM-150 µM), especially in the 100 µM and 150 µM co-exposure GA groups ($P<0.05$) (Figure 5A).

Discussion

The present study aimed to identify the molecular mechanism and critical signaling pathways by which GA produces a potential protective effect against NiSO$_4$ toxicity. The results from our study suggest that NiSO$_4$ induced cytotoxicity, oxidative stress, and apoptosis through the Ras/ERK pathway in human bronchial epithelial Beas-2B cells. Addition of GA at the certain safe doses obviously inhibited NiSO$_4$-induced cell injury and also reduced the expression of Ras/ERK signaling protein, particularly in the 100 µM and 150 µM co-exposure GA groups.

Figure 5. Effect of GA on NiSO$_4$-induced Ras/ERK signaling pathway protein expressions. Shown are expression levels of Ras/ERK signaling pathway proteins after Beas-2B cells were treated with NiSO$_4$ alone or NiSO$_4$ + GA. (**A**) NiSO$_4$ regulated Ras/ERK signaling protein expressions in Beas-2B cells. Cells were exposed to different concentrations of NiSO$_4$ for 24 h; effect of GA on NiSO$_4$-induced Ras/ERK signaling pathway protein expressions. Cells were exposed to 500 μM NiSO$_4$ for 24 h in the presence or advance of GA (0–150 μM). (**B**) Beas-2B cells were not treated or treated with Ras inhibitor manumycin A (10 μM) or ERK inhibitor PD98059 (20 μM) for 1 h, and then with 500 μM NiSO$_4$ for 24 h. The protein expression levels were determined by Western blotting analysis as described in the Material and Methods section.

It is well known that the body is in the equilibrium state of oxidation and antioxidation under normal physiological conditions. Studies in recent years, however, have shown that nickel ions through the Fenton reaction cause an oxidative stress reaction in the body, generating ROS and producing adverse biological effects in cells, including cell membrane damage and apoptosis [16]. LPOs, including MDA, LDH, and the others, can reflect the change of oxidative stress. By measuring these oxidative stress parameters, we found that the level of GSH decreased with the increase of ROS and LPO levels after NiSO$_4$ exposure. By adjusting the LPO index changes in Beas-2B cells, GA, as a kind of antioxidant extracted from plants, effectively reduced the oxidative stress effect caused by NiSO$_4$, thus reducing the damage. Our research found that GA can be a contributing factor to lipid peroxidation, evidenced by MDA and LDH levels that were significantly decreased in the

co-exposure GA groups when compared to the NiSO$_4$ treatment group. As an antioxidant, GA widely exists in grapes, pomegranates, black tea, and traditional Chinese medicine. Studies have confirmed that GA through the Fenton reaction produces a hydroxyl radical and xanthine oxidase free-radical system, resulting in superoxide anion free-radical scavenging, and that GA has an inhibitory effect on human liver microsomal cytochrome P450 3A (CYP3A) mediated oxidation, to reduce the tissue accumulation of ROS. In the induction of apoptosis, GA mainly had a pro-oxidative effect. We also demonstrated that GA significantly protected against the oxidative stress induced by NiSO$_4$ in Beas-2B cells.

Apoptosis is a physiological mechanism that plays a very important role in maintaining the stability of the organismic internal environment, but when apoptosis is too high or too low,

it will have adverse effects on the body. A large number of studies have shown that oxidative stress may induce apoptosis [17,18]. Our observation showed that $NiSO_4$ induced apoptosis accompanied by LPO level changes (see Supplementary Figures 1–3). ROS can initiate a chain reaction, which is easy to react with various kinds of unsaturated fatty acids and cholesterol in the cell membrane. Previous studies have demonstrated that nickel ion-induced oxidative stress leads to apoptosis [19–23]. There is a close link between the development of oxidative stress and apoptosis [24]. Before $NiSO_4$ infected Beas-2B cells, GA intervention could significantly inhibit apoptosis caused by $NiSO_4$. By reducing the increased level of ROS and apoptosis rate, GA inhibited the cell injury, and therefore has respiratory protective actions after $NiSO_4$ exposure.

In order to investigate the specific molecular mechanism of the protective effect on human bronchial epithelial Beas-2B cells apoptosis induced by GA, we further detected the expression levels of Ras/ERK signaling pathway proteins, including Ras, ERK, c-Myc, etc. The Myc oncogene family is considered to be related to the occurrence of many kinds of malignant diseases [25], in which the c-Myc gene encoding protein is a transcription factor, which is expressed in most human diseases [13]. Previous studies also have shown that there were several potential mechanisms of c-Myc-induced apoptosis, including the increase of DNA strand breaks and the decrease of bcl-2 expression [26,27]. In recent years, particular attention has been paid to studies on the multiple pathways that control c-Myc, such as NF-κB [24], Ras/JNK [28], and Ras/ERK [29]. Li et al. reported that nickel ions induced c-Myc in Beas-2B cells by the Ras/ERK pathway [3]. Western blot analysis results indicated that 250~1000 μM $NiSO_4$ significantly increased the protein levels of Ras, p-ERK, and c-Myc (Figure 5). Ras/ERK signaling pathway inhibitors were used to confirm whether the Ras/ERK pathway may be related to $NiSO_4$-induced c-Myc. The results showed that both Ras and ERK inhibitors reduced $NiSO_4$-induced

c-Myc protein. Meanwhile, 500 μM-1000 μM $NiSO_4$ significantly increased PARP cleavage, which represented a marker of apoptosis. After GA intervention, the expression levels of c-Myc–related proteins were suppressed. Especially in the 100–150 μM GA co-exposure group, GA down-regulated the expression levels of c-Myc and PARP cleavage. These findings demonstrate that GA has a protective effect against $NiSO_4$-induced apoptosis via inhibiting the Ras/ERK signaling pathway.

To sum up, the results of our study suggested the safe dose of GA in Beas-2B cells is 10–150 μM. The protective effect of GA against $NiSO_4$-induced cell toxicity in Beas-2B cells might be through the Ras/ERK pathway, which indicates that GA has an effect on preventing cell injury.

Conclusions

In this study, we found that GA could reduce $NiSO_4$-induced toxicity through down-regulation of the Ras/ERK signaling pathway in Beas-2B cells. The limitation is that the protective effect of GA on $NiSO_4$-induced cell toxicity was only evaluated by *in vitro* experiments; however, there have been relatively few systematic *in vivo* studies involving the mechanism of the protective effect of GA. Thus, it has not been possible to identify whether the activation of Ras/ERK signaling pathway is the direct manifestation of toxicity or whether it is the protective response of the cells toward toxic substances. In order to provide a sufficient basis for identification of the mechanism of the protective effect of GA when exposed to toxic substances, further studies are needed to elucidate this relationship.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Supplementary Figures
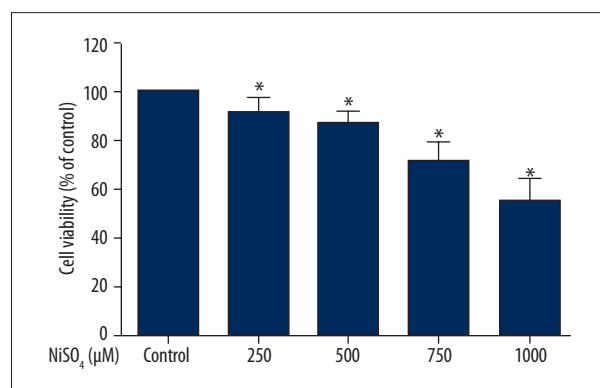
Supplementary Figure 1. $NiSO_4$ induced cytotoxicity in Beas-2B cells. Cells were exposed to different concentrations of $NiSO_4$ for 24 h, and cell viability was determined by MTT assay as described in the Material and Methods section. Data represent mean ±SD of three independent experiments made in three replicates. * $P<0.05$, compared with control group.
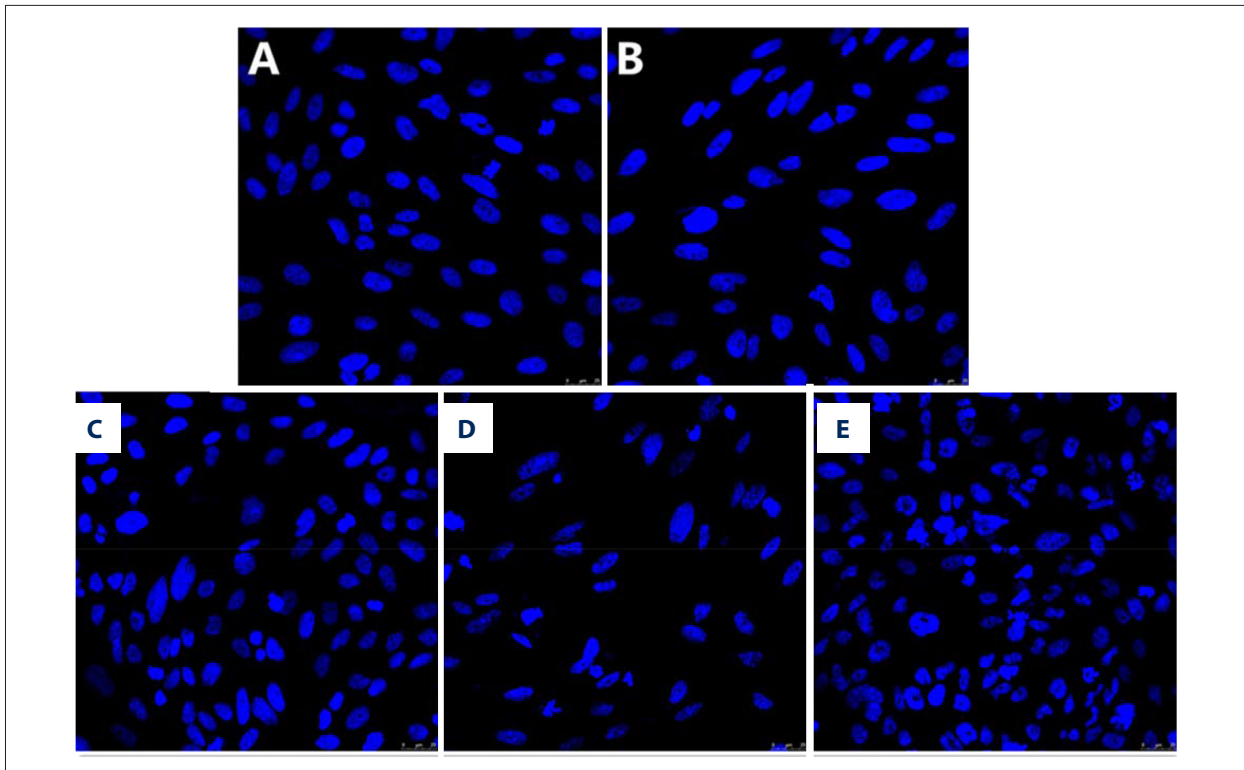
Supplementary Figure 2. $NiSO_4$ induced cytotoxicity in Beas-2B cells. Nuclear staining of Beas-2B cells was with Hoechst 33258. (**A**) Control group. (**B**) 250 μM $NiSO_4$. (**C**) 500 μM $NiSO_4$. (**D**) 750 μM $NiSO_4$. (**E**) 1000 μM $NiSO_4$. The original magnification is 40×.
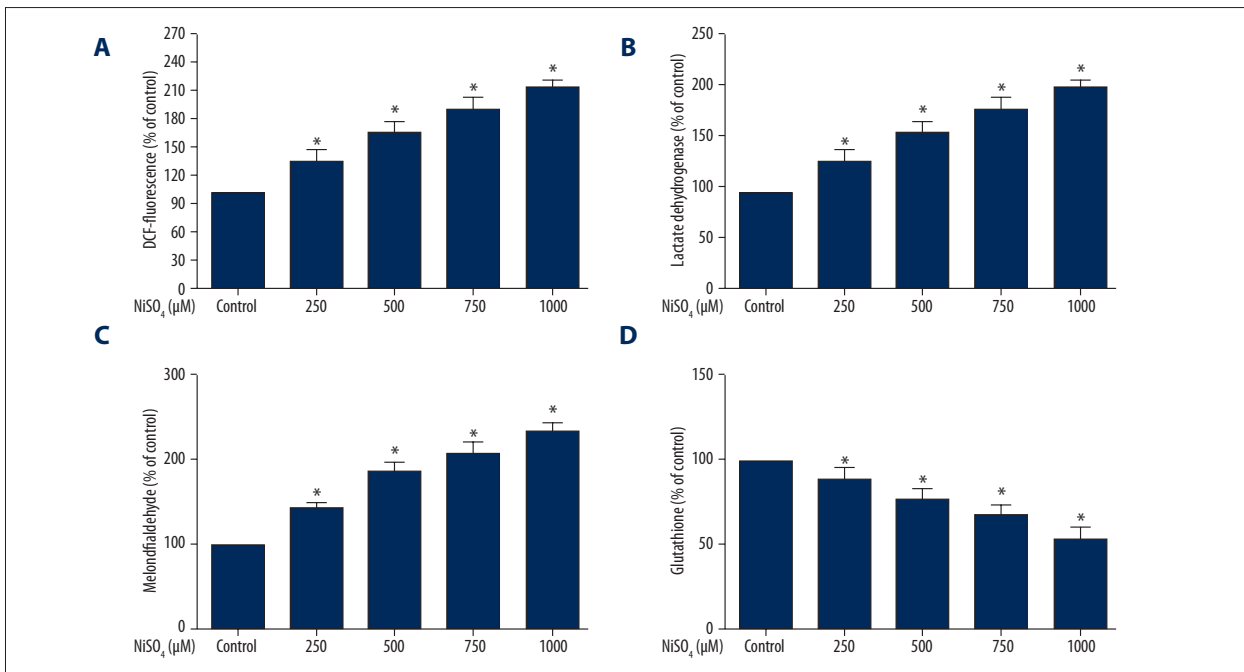
Supplementary Figure 3. $NiSO_4$ induced oxidative stress in Beas-2B cells. Cells were exposed to different concentrations of $NiSO_4$ for 24 h, and cells' oxidative stress parameters were determined according to the kit instructions as described in the Material and Methods section. (**A**) ROS, (**B**) LDH, (**C**) MDA, (**D**) GSH. Data represent mean ±SD of three independent experiments made in three replicates. * P<0.05, compared with control group.

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