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Ephedrannin B exerts anti-viral and anti-inflammatory properties in BEAS-2B cells infected with respiratory syncytial virus

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Ephedrannin B (EPB) has been shown to exert anti-inflammatory effects. However, the effect of EPB on respiratory syncytial virus infection (RSV) is not known. In this study, the cytotoxic effect of EPB was evaluated in BEAS-2B cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Reverse transcription quantitative polymerase chain reaction and Western blot assays were performed to determine the expression of target genes. The anti-viral effect of EPB was assessed by determining viral titers using plaque assay. We found that RSV infection caused a marked increase in interleukin (IL)-6, IL-8, IL-1β and tumor necrosis factor (TNF)-α production and release, which was concentration-dependently attenuated by EPB treatment. Furthermore, EPB decreased the expression of RSV fusion gene in RSV-infected BEAS-2B cells. Concomitantly, EPB treatment led to an obvious inhibition of viral replication in BEAS-2B cells. Besides, EPB suppressed RSV-induced mitogen-activated protein kinase/nuclear factor kappa-light-chain-enhancer of activated B cells signaling. In conclusion, EPB exerts anti-viral and anti-inflammatory properties in BEAS-2B cells infected with RSV.

Keywords. Ephedrannin B; inflammatory cytokines; respiratory syncytial virus; viral titers

1. Introduction

Respiratory syncytial virus (RSV), a negative-sense RNA virus belonging to the paramyxoviridae family, is generally recognized as the most common respiratory pathogen in children (Lozano *et al.* 2012). RSV is the principal etiological factor of lower respiratory tract infections and is the leading cause of death in young children, contibuting to approximately 2,00,000 child deaths worldwide annually (Jansen *et al.* 2007; Nair *et al.* 2010). Since the organism cannot develop a persistent immune reaction to RSV infection, RSV infection recurs easily (Chen *et al.* 2018). Currently, there is no safe and valid vaccine or medicine for RSV (Schmidt and Varga 2017). Therefore, an effective anti-RSV drug in clinical practice is sought urgently.

It is generally known that RSV infection is usually accompanied by an inflammatory response. During RSV infection, RSV binds to pattern recognition receptors and then activates the immune system to trigger an inflammatory response, resulting in the recruitment of immune cells (Russell *et al.* 2017). There is no doubt that appropriate inflammatory responses can facilitate the efficient clearance of the virus and repair the destroyed tissue caused by RSV. However, the exaggerated inflammation, also called the 'cytokine storm', causes tissue destruction, ultimately initiating bronchiolitis or pneumonia (Openshaw *et al.* 2017). Hence, suppression of RSV-induced inflammation is of great importance for RSV therapy.

Ephedra sinica Stapf, an important Chinese medicinal herb, has wide pharmacological effects, such as anti-inflammatory, anti-microbial, anti-obesity and anti-tumor effects (Hyuga 2017; Lee *et al.* 2010; Oh *et al.* 2015; Wang *et al.* 2016). In addition, extracts of *Ephedra sinica Stapf* were reportedly capable of

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stimulating the sympathetic nervous system, dilating bronchial tubes and elevating blood pressure (Andraws *et al.* 2005; Ma *et al.* 2007). Although the application of *Ephedra sinica Stapf* is well established in the clinic, the mechanism of its pharmacological effects have not been defined in depth. Recently, emerging evidence suggests that the pharmacological effects of *Ephedra sinica Stapf* are closely relevant to their active medicinal ingredients, including Ephedrannin B (EPB). EPB is an A-type proanthocyanidine isolated from the roots of *Ephedra sinica Stapf.* EPB has been documented to be regarded as a major active ingredient of *Ephedra sinica Stapf*, as in the case of its anti-tumor effects in SGC-7901, HepG2 and HeLa cells (Tao *et al.* 2008). Moreover, a previous study in melanoma B16F10 cells suggested that EPB repressed the generation of melanin through repressing the transcription of tyrosinase (Kim *et al.* 2015). However, the effect of EPB on RSV infection has not been previously elucidated. Both Ephedrannin A (EPA) and EPB have been shown to exert anti-inflammatory activity, and the inhibition by EPB was much more effective than that by EPA (Kim *et al.* 2010). Immoderate inflammation plays a great role in causing RSV-induced diseases, including acute airway obstruction, airway inflammation and lung inflammation. Therefore, we speculated that Ephedrannin B might restrain RSV-induced inflammation.

In the present study, we investigated the effect of EPB on RSV infection in BEAS-2B cells. Further, we determined the impact of EPB on inflammatory cytokines production, RSV fusion (RSV-F) mRNA expression and mitogen-activated protein kinase (MAPK)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in BEAS-2B cells to clarify the anti-inflammation and anti-RSV mechanism of EPB. Our findings revealed that EPB exerts anti-viral and anti-inflammatory properties in BEAS-2B cells infected with RSV by regulating the MAPK/NF-κB signaling pathway.

2. Materials and methods

2.1 *Virus preparation, cell culture, and RSV infection in vitro*

Human RSV virus strain A2 was propagated in Hep-2 cells (American Type Culture Collection, Rockville, MD) with culturing in Dulbecco's minimal essential medium (DMEM; Solarbio, Beijing, China), supplemented with 10% fetal calf serum (FCS; Solarbio). After 1 h of adsorption, Hep-2 cells were washed with PBS and cultured in fresh medium for 48 h. After centrifugation, cell supernatants were collected and stored at −80°C.

BEAS-2B cells (ATCC) were incubated in DMEM containing 10% FCS for 36 h and infected for indicated time (12 and 24 h) with RSV at a multiplicity of infection of 0.5. Following this, BEAS-2B cells were treated with indicated dose (0, 5 and 10 μM) of EPB (Chemfaces, Wuhan, China) for 48 h.

2.2 *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*

BEAS-2B cells (1×10^4 cells/well) were seeded in a 96-well plate, and then exposed to increasing dose (0, 1, 2.5, 5, 10, 20 and 40 μM) of EPB for 48 h. After treatment, cells were incubated with 10 μl MTT solution (Solarbio) for 4 h under 5% CO_2/95% air at 37°C. Following this, formazan solution (110 μl; Solarbio) was added into each well and cultured on a shaker for 10 min. The absorbance of each well at the wavelength of 490 nm was determined using a microplate reader.

2.3 *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Total RNA was extracted from BEAS-2B cells using an RNA extraction kit (BioTeke, Beijing, China), followed by reverse transcription to cDNA using SuperScript First-Stand Synthesis system (Invitrogen, Carlsbad, CA, USA). RT-qPCR assay was conducted on a StepOnePlus® Real-Time PCR System (Applied Biosystems, Warrington, UK) using SYBR Premix Ex Taq™ kit (Takara, Dalian, China) with the following primers: RSV-F forward, 5′-TGC AGT GCA GTT AGC AAA GG-3′ and reverse, 5′-TCT GGC TCG ATT GTT TGT TG-3′; interleukin (IL)-6 forward, 5′-ATG AAC TCC TTC TCC ACA AGC GC-3′ and reverse, 5′-GAA GAG CCC TCA GGC TGG ACT G-3′; IL-8 forward, 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′ and reverse, 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′; IL-1β forward, 5′-ATG GCA GAA GTA CCT AAG CTC GC-3′ and reverse, 5′-ACA CAA ATT GCA TGG TGA AGT CAG TT-3′; Tumor necrosis factor (TNF)-α forward, 5′-ATG AGC ACT GAA AGC ATG ATC CGG-3′ and reverse, 5′-GCA ATG ATC CCA AAG TAG ACC TGC CC-3′; β-actin forward, 5′-TGA CGT GGA CAT CCG CAA AG-3′ and reverse, 5′-CTG GAA GGT GGA CAG CGA GG-3′. The expression of target genes was

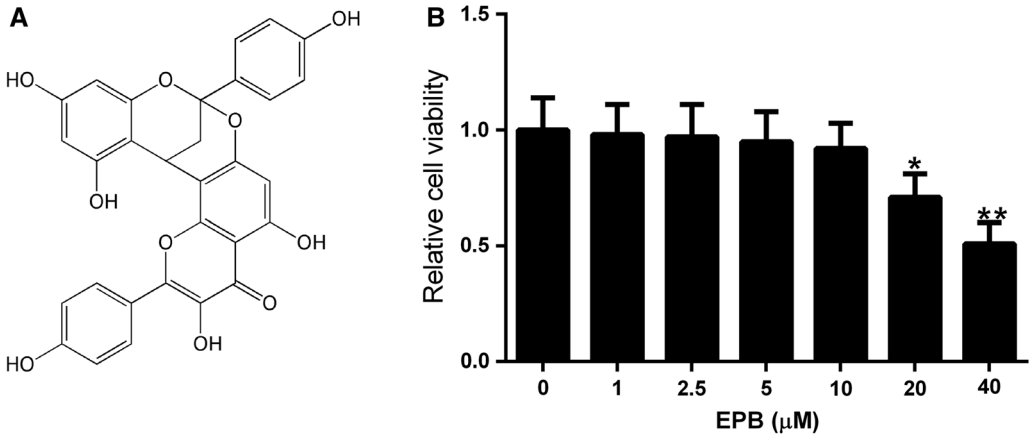

Figure 1. Cytotoxic effect of EPB on BEAS-2B cells. (**A**) The structure of EPB. (**B**) BEAS-2B cells were treated with different concentrations (0, 1, 2.5, 5, 10, 20 and 40 μM) of EPB, and then subjected to MTT assay. *$P < 0.05$, *vs* Control group.

analyzed by $2^{-\Delta\Delta CT}$ method using β-actin as an internal control.

2.4 *Cell supernatant analysis*

Enzyme-linked immunosorbent assay (ELISA; Beyotime, Shanghai, China) was performed to examine the release of IL-6, IL-8, IL-1β, TNF-α in BEAS-2B cell supernatants, as directed by manufacturer's specifications.

2.5 *Plaque assay*

BEAS-2B cells (~90% confluent) were seeded on a 12-well plate and then infected with RSV at a multiplicity of infection of 0.5 for 24 h, with shaking every 15 min. After infection, BEAS-2B cells were rinsed with PBS to remove RSV. Following this, BEAS-2B cells were cultured for 4–5 days in fresh medium containing 0.9% methylcellulose under 5% CO_2/95% air at 37°C and syncytia were allowed to form. Afterwards, BEAS-2B cells were rinsed thrice with PBS, fixed with 3% paraformaldehyde (Solarbio) for 30 min and stained with 1% crystal violet (Solarbio) for 30 min. The number of virus-induced plaques formed was counted under a light microscope.

2.6 *Western blot*

After treatment, BEAS-2B cells were collected and lysed with RIPA buffer containing protease inhibitors (Sigma-Aldrich, Louis, MO, USA). After resolution on 14% sodium dodecyl sulfate polyacrylamide gel electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes, followed by blockade with 5% non-fat milk for 1 h at room temperature. Membranes were probed overnight at 4 °C with primary antibodies against p38, phospho-p38 (p-p38), extracellular signal-regulated kinases (ERK), phospho-ERK (p-ERK), p65, phospho-p65 (p-p65) and β-actin, followed by incubation for 1 h at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies. Bands were visualized with the ECL system (Pierce, Rockford, IL, USA). All antibodies were procured from Abcam (Cambridge, UK).

2.7 *Statistical analysis*

All data were presented as the mean ± standard deviation of the mean (SD) and analyzed using SPSS 20.0 software (SPSS Inc., Chicago, Illinois, USA). Statistical significance was processed by use of one-way analysis of variance. Each experiment was performed at least three times and $P < 0.05$ was defined as the limit of statistical significance.

3. Results

3.1 *Cytotoxic effect of EPB on BEAS-2B cells*

To determine whether EPB is cytotoxic to BEAS-2B cells, we exposed BEAS-2B cells to different doses

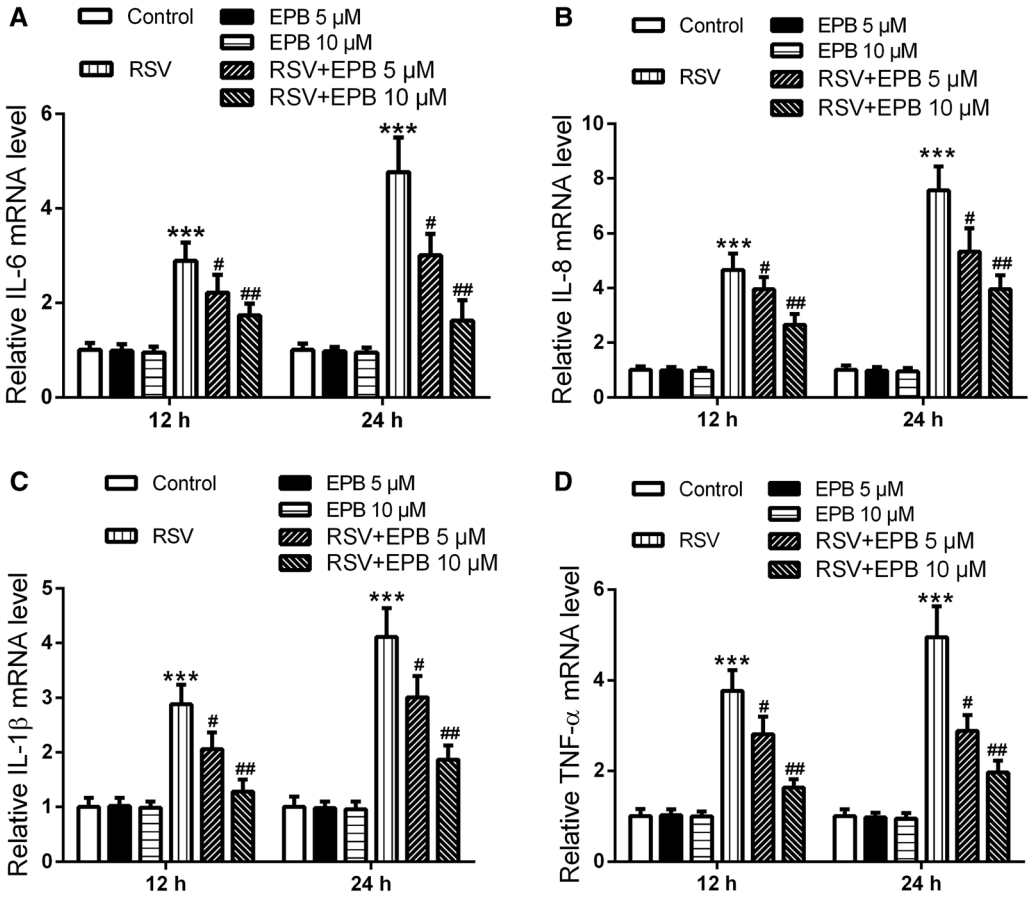

Figure 2. Inhibitory effect of EPB on the production of inflammatory cytokines from RSV-infected BEAS-2B cells. BEAS-2B cells were infected with RSV for indicated time (12 and 24 h), and then treated with different doses (0, 5 and 10 μM) of EPB for 48 h. The mRNA expression levels of IL-6 (**A**), IL-8 (**B**), IL-1β (**C**) and TNF-α (**D**) in different groups were determined by RT-qPCR. ***$P < 0.001$, *vs* Control group; #$P < 0.05$, ##$P < 0.01$, *vs* RSV group.

(0, 1, 2.5, 5, 10, 20 and 40 μM) of EPB and then subjected to MTT assay. Treatment of BEAS-2B cells with EPB (0, 1, 2.5, 5 and 10 μM) did not affect cell viability, while the viability of BEAS-2B cells was markedly reduced when cells were treated with 20 and 40 μM of EPB (figure 1B). Therefore, these two concentrations (5 and 10 μM) of EPB were applied in the subsequent experiments.

3.2 *Inhibitory effects of EPB on the production of inflammatory cytokines from RSV-infected BEAS-2B cells*

The results showed that no changes were found in the levels of inflammatory cytokines from BEAS-2B cells treated with EPB alone (5 and 10 μM) (figure 2A–D). Subsequently, the effect of EPB on RSV-induced inflammation was explored in vitro. BEAS-2B cells were infected with RSV for indicated time (12 and 24 h), and then treated with different doses (0, 5 and 10 μM) of EPB. We examined the mRNA expression of IL-6, IL-8, IL-1β and TNF-α in BEAS-2B cells and found that RSV infection strikingly increased the mRNA expression levels of IL-6, IL-8, IL-1β and TNF-α. Of note, EPB treatment dose-dependently reduced the mRNA expression levels of IL-6, IL-8, IL-1β and TNF-α in RSV-infected BEAS-2B cells (figure 2A–D).

3.3 *Inhibitory effects of EPB on the release of IL-6, IL-8, IL-1β and TNF-α in BEAS-2B cell supernatants*

To investigate whether EPB affects the release of inflammatory cytokines induced by RSV, we measured the concentrations of IL-6, IL-8, IL-1β and TNF-α in the cell supernatants from RSV-infected BEAS-2B cells using ELISA assay. As shown in figure 3, RSV

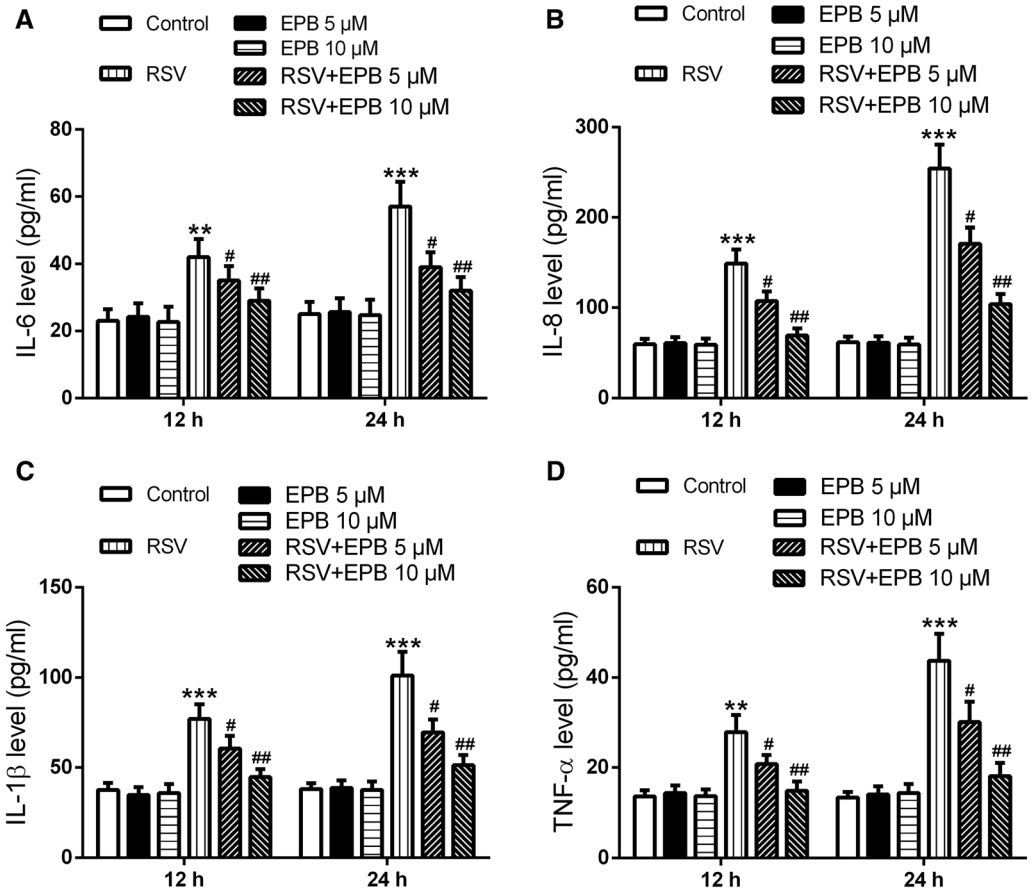

Figure 3. Inhibitory effect of EPB on the release of IL-6, IL-8, IL-1β and TNF-α in BEAS-2B cell supernatants. BEAS-2B cells were infected with RSV for indicated time (12 and 24 h), and then treated with different doses (0, 5 and 10 μM) of EPB for 48 h. Treatment of BEAS-2B cells with EPB strongly inhibited the release of IL-6 (**A**), IL-8 (**B**), IL-1β (**C**) and TNF-α (**D**) induced by RSV infection. ***$P < 0.001$, *vs* Control group; #$P < 0.05$, ##$P < 0.01$, *vs* RSV group.

infection strikingly increased the levels of IL-6 (figure 3A), IL-8 (figure 3B), IL-1β (figure 3C) and TNF-α (figure 3D) release in the cell supernatants from RSV-infected BEAS-2B cells, which were dose-dependently mitigated by EPB treatment.

3.4 *Anti-viral activity of EPB in RSV-infected BEAS-2B cells*

To study the anti-viral effect of EPB, BEAS-2B cells were infected with RSV and treated with different doses (0, 5 and 10 μM) of EPB. As determined by RT-qPCR assay, the expression of RSV-F was elevated after RSV infection, and EPB administration dose-dependently blocked RSV-induced elevation of RSV-F expression (figure 4A). Likewise, a plaque assay revealed that EPB treatment reduced the number of virus-induced plaques formed, in a dose-dependent manner (figure 4B).

3.5 *Inhibitory effects of EPB on the MAPK and NF-κB signaling pathways in RSV-infected BEAS-2B cells*

The results of Western blot showed that treatment with EPB alone (5 and 10 μM) did not affect the MAPK and NF-κB signaling pathways in BEAS-2B cells. RSV infection activated the MAPK and NF-κB signaling pathways *in vitro* (figure 5B). Next, we investigated whether EPB could affect the activation of the MAPK and NF-κB signaling pathways in RSV-infected BEAS-2B cells. The results of western blot revealed that the protein expression levels of the MAPK signaling-related molecules, such as p-p38 MAPK, p-ERK and p-p65, were obviously increased in RSV-infected BEAS-2B cells, however, this action was mitigated by EPB treatment (figure 5A). Similarly, RSV infection activated the NF-κB signaling pathway, indicated by enhanced phosphorylation of p65. However, treatment of EPB decreased the phosphorylation

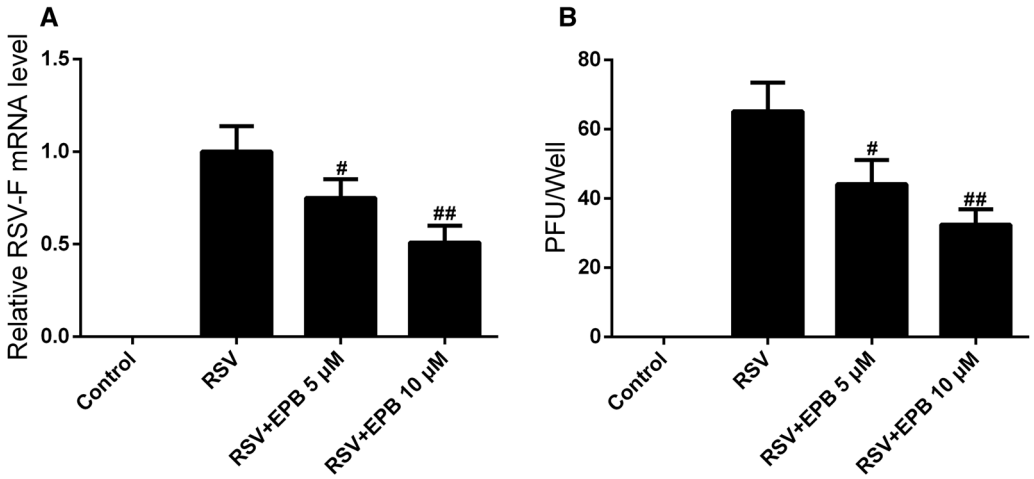

Figure 4. Anti-viral activity of EPB in RSV-infected BEAS-2B cells. BEAS-2B cells were infected with RSV for 24 h, followed by treatment with different doses (0, 5 and 10 μM) of EPB. (**A**) RT-qPCR analysis of RSV-F expression showed reduced expression of RSV-F in RSV-infected BEAS-2B cells in the presence of EPB (5 and 10 μM). (**B**) At 48 h after EPB treatment, RSV titer was assessed by plaque assay. #$P < 0.05$, ##$P < 0.01$.

level of p65 in RSV-infected BEAS-2B cells (figure 5B).

4. Discussion

Although *Ephedra sinica Stapf* has been used extensively in clinical treatment, surprisingly little attention has been devoted to the pharmacological activities of its active ingredient, EPB, especially in RSV infection. In this study, we investigated the biological activity of EPB in RSV infection. Our findings revealed that EPB exerted anti-viral and anti-inflammatory properties BEAS-2B cells infected with RSV, suggesting that EPB may be a novel effective agent for combating RSV infection.

RSV-F, a transmembrane protein, is required for mature particle formation and plays an obligatory role in RSV infection and replication (Pierangeli *et al.* 2018; Tang *et al.* 2018). RSV-F has been shown to mediate cell-fusion with the virus and the target cell membrane, and facilitate the formation of syncytium between neighboring cells (Canedo-Marroquin *et al.* 2017). The significance of RSV-F in RSV infection has been extensively studied, and targeting RSV-F appears to be a promising approach to protect against RSV infection (Khan *et al.* 2018; Tang *et al.* 2018). In this study, RSV-F expression was markedly increased in BEAS-2B cells following RSV infection, however, this increase was dose-dependently blocked by EPB treatment. Moreover, treatment of BEAS-2B cells with EPB dose-dependently inhibited RSV-induced plaque formation. These data indicated that EPB treatment efficiently inhibits the replication of RSV in BEAS-2B cells.

Apart from the direct pathogenicity of RSV, inflammation is also recognized as a central contributor to RSV-induced tissue damage (Smallcombe *et al.* 2018; van Erp *et al.* 2018). Interestingly, Kim and colleagues have been identified the structure and anti-inflammatory activity of EPB in lipopolysaccharide-stimulated RAW 264.7 cells. They showed that EPB treatment markedly inhibited the production of NO, TNF-α, and IL-1β induced by lipopolysaccharide through inhibiting the activation of the NF-κB and MAPK signaling (Kim *et al.* 2010). However, whether EPB protects against RSV-induced inflammation has not been studied yet. Our data suggested that treatment with EPB alone had no effect on the production of inflammatory factors in BEAS-2B cells. The levels of IL-6, IL-8, IL-1β and TNF-α were increased in RSV-infected BEAS-2B cells, as well as in cell supernatants from RSV-infected BEAS-2B cells. However, these changes caused by RSV infection were dose-dependently abated by EPB treatment, raising the possibility that EPB can attenuate RSV-induced inflammation in vitro.

The MAPK and NF-κB signaling pathways have been proposed to play a central role in the eukaryotic stress responses, including virus infection (Li *et al.* 2018). Previous studies have noted that the MAPK and NF-κB signaling pathways are implicated in the process of RSV infection. For instance, studies in macrophages showed that RSV infection caused the activation of the canonical and non-canonical NF-κB

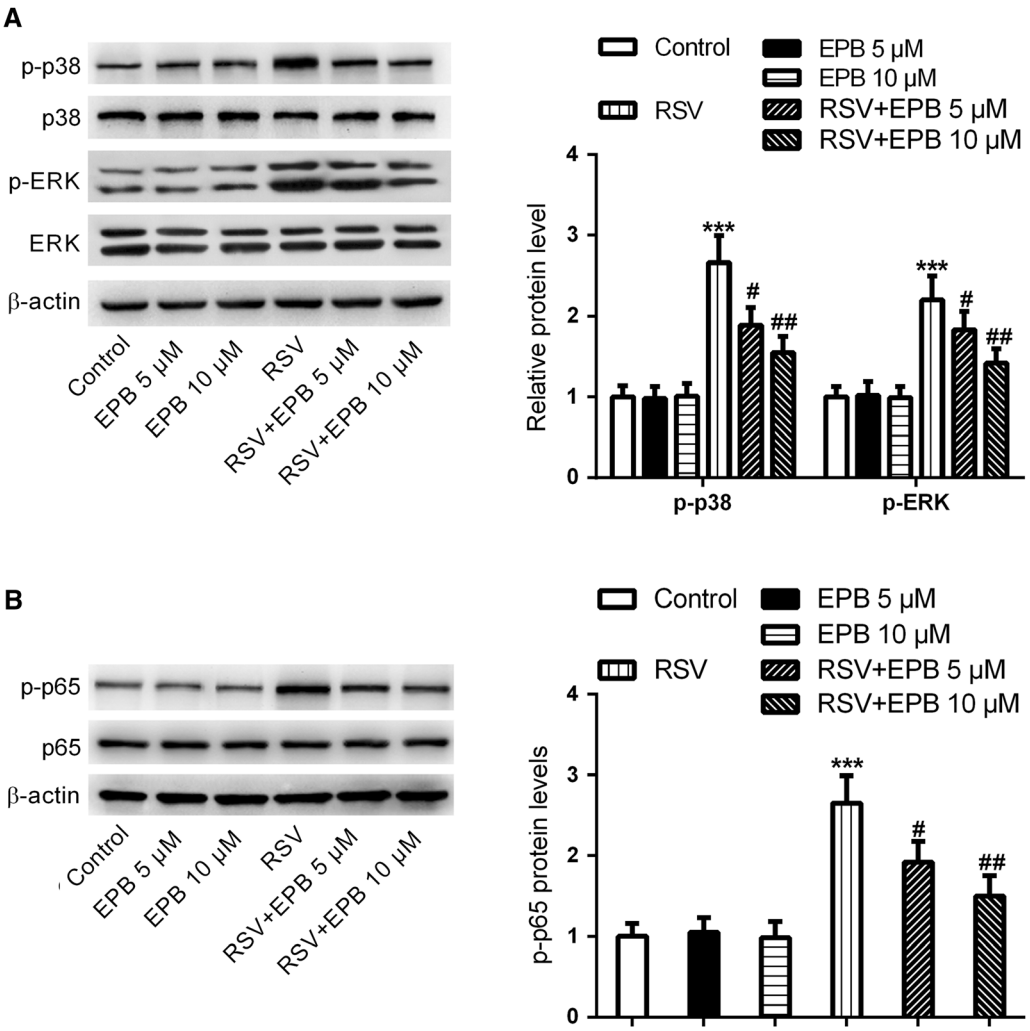

Figure 5. Changes in p-p38, p-38, p-ERK, ERK, p-p65 and p65 protein expression in RSV-infected BEAS-2B cells in the presence of EPB. BEAS-2B cells were infected with RSV for 24 h, followed by treatment with different doses (0, 5 and 10 μM) of EPB. (**A**) Western blot analysis of p-p38, p38, p-ERK and ERK expression (the MAPK signaling pathway) in RSV-infected BEAS-2B cells in the presence of EPB. (**B**) Western blot analysis of p-p65 and p65 expression (the NF-κB signaling pathway) in RSV-infected BEAS-2B cells in the presence of EPB. ***$P < 0.001$, *vs* Control group; #$P < 0.05$, ##$P < 0.01$, *vs* RSV group.

signaling and the upregulation of pro-inflammatory cytokines, such as p52, IL-1β, TNF-α and chemokine (C–C motif) ligand 5 (Moral-Hernandez *et al.* 2018). Additionally, treatment of RSV-infected A549 cells with grape seed proanthocyanidin resulted in an obvious inhibition of mucin synthesis and viral replication by repressing AP-1 and NF-κB via inhibition of p38 MAPKs/c-Jun N-terminal kinases signaling activation (Lee *et al.* 2017). However, whether the MAPK and NF-κB signaling pathways are implicated in the anti-RSV activity of EPB remains incompletely clear. Herein, we showed that treatment with EPB alone had no effect on the MAPK and NF-κB signaling pathways in BEAS-2B cells. RSV infection caused an increase in p-p38, p-ERK and p-p65 expression, which was dose-dependently mitigated by EPB treatment, suggesting the involvement of MAPK and NF-κB signaling in the anti-RSV activity of EPB. In our future research, we will further investigate the anti-viral and anti-inflammatory properties of EPB with a positive control in a mouse model of respiratory syncytial virus infection. The anti-viral and anti-inflammatory efficiencies of ephedrannin B and the positive control drug will also be compared.

In summary, we identified EPB as a potential therapeutic drug that shows anti-viral and anti-inflammatory properties in BEAS-2B cells infected with RSV. Moreover, the MAPK and NF-κB signaling pathways were identified to be involved in the anti-RSV activity of EPB. Our findings suggest that EPB promises to be a

therapeutic agent for combating RSV infection, providing a theoretical basis for its clinical practice.

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