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Original Research Article

Chrysoeriol alleviated inflammation in infantile pneumonia by inhibiting PI3K/AKT/mTOR signaling pathway

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Abstract

Purpose: *To assess the therapeutic effects of chrysoeriol (CHE) on pediatric pneumonia and determine the mechanism of action.*
Methods: *The role of chrysoeriol was investigated in a human lung fibroblasts (HFL1) cell model of pneumonia. The effects of lipopolysaccharide (LPS) and CHE on cell viability and apoptosis were determined by CCK-8 kit and flow cytometry, respectively, while oxidative stress was determined by evaluating the levels of superoxide dismutase (SOD), glutathione, r-glutamyl cysteine +glycine (GSH)-px, myeloperoxidase (MPO) and malondialdehyde (MDA). Inflammatory response was assessed by determining IL-1β, TNF-α, IL-18 and IL-10 levels using enzyme-linked immunosorbent assay (ELISA). The mechanisms of action of CHE were evaluated by immunoblot assays.*
Results: *Chrysoeriol increased the viability of LPS-induced pneumonia cells ($p < 0.001$) but decreased cell apoptosis ($p < 0.001$). Furthermore, chrysoeriol reduced oxidative stress and inflammation in LPS-induced pneumonia cells, and suppressed the activation of PI3K/AKT/mTOR pathway.*
Conclusion: *Chrysoeriol alleviates inflammation of infantile pneumonia by inhibiting PI3K/ AKT /mTOR signaling pathway. Thus, CHE is a potential drug for the management of pneumonia.*

Keywords: *Chrysoeriol, Pneumonia, Cell viability, Oxidative stress, PI3K/AKT/mTOR pathway*

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INTRODUCTION

Infantile pneumonia is a common disease and a common cause of infant death [1]. The World Health Organization (WHO) estimates there are 150.7 million cases of lung infections in children under the age of five each year [2]. Pediatric pneumonia is one of the common diseases in pediatrics, and is caused by bacteria and viral infections. The main symptoms are dyspnea, fever, cough and shortness of breath. It is a common cause of death in infants and young children due to its duration and severe symptoms, and may induce a variety of extrapulmonary complications [3]. To further alleviate these symptoms and improve patient outcomes and survival prospects, new and more effective treatments are needed.

Chrysoeriol is a flavonoid found in a variety of dietary and medicinal materials [4]. These herbs are commonly used to treat inflammatory

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diseases [5]. Honeysuckle effectively prevents acute lung inflammation by decreasing the levels of pro-inflammatory factors in serum and bronchoalveolar lavage fluid, as well as the levels of oxidative stress factors in lung tissue [6]. These studies suggest that chrysoeriol might play a critical role in the treatment of lung-related diseases.

Chrysoeriol has been proven to exert a variety of pharmacological properties, such as anti-inflammatory, antioxidant, anti-tumor, smooth muscle relaxation, obesity reduction, and immune system regulation [7]. Chrysoeriol induced apoptosis of rat glioma cells via the suppression of PI3K/Akt/mTOR pathway [8]. In addition, chrysoeriol ameliorates TPA-induced skin inflammation in mice by inhibiting NF-κB and STAT3 pathways [9]. In lipopolysaccharide (LPS)-induced mouse macrophages, chrysoeriol significantly inhibited PGE2 secretion and COX-2 expression, as well as Akt and P38 phosphorylation, but had no significant effect on ERK phosphorylation [10]. Studies on the therapeutic effect and mechanism of chrysoeriol in children with pneumonia have not been reported. In this study, the effect of chrysoeriol was investigated in a cell model of pneumonia.

EXPERIMENTAL

Cell culture

Human lung fibroblasts (HFL1) were obtained from the Cell Resource Center of Peking Union Medical College (Beijing, China), and incubated with Dulbecco's modified Eagle's medium (DMEM, Gibco, Thermo Fisher, USA) containing 10 % FBS, and 1 % penicillin-streptomycin in a humidified culture hood with 5 % CO_2 at 37 °C. For LPS induction, 1 μg/mL LPS was added to the cells. The cells were pre-treated with 5, 10 or 15 μM chrysoeriol (CHE), or 0.1 % DMSO (control) for 2 h; thereafter, LPS was added for another 24 h prior to further evaluation.

Cell counting kit-8 (CCK-8) assay

To assess cell viability, CCK-8 assay was performed. After the indicated treatments, CCK-8 reagent to HFL1 cells in each well and cultured for 1 h at 37 °C, following which the absorbance of each well was read with a microplate reader at 450 nm.

Flow cytometry

Cells were placed in a 96-well plate and treated. Thereafter, the cells were fixed and labeled with 50 μg/mL propidium iodide for 30 min in the dark at room temperature. The stained cells were washed by centrifugation, re-suspended and analyzed on a BD FACSCanto II (BD Biosciences).

Western blotting

Cell lysates were collected after RIPA buffer addition. After centrifugation, protein concentration was determined with BCA kit (Beyotime Biotechnology, Shanghai, China). The proteins were subjected to 10 % SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes, which were then incubated with 5 % BSA, followed by primary antibodies targeting Bcl-2 (1:1000, Abcam, Cambridge, UK), Bax (1:1000, Abcam, Cambridge, UK), PARP (1:1000, Abcam), cleaved-PARP (1:1000, Abcam), p-PI3K (1:1000, Abcam), PI3K (1:1000, Abcam), p-AKT (1:1000, Abcam), AKT (1:1000, Abcam), p-mTOR (1:1000, Abcam), mTOR (1:1000, Abcam), and GAPDH (1:10000, Abcam) for 2 h at room temperature.

The membranes were maintained in Horseradish Peroxidase (HRP)-conjugated secondary antibodies at a 1:1000 for 2 h after rinsing in TBST for 15 min at room temperature. The signals were detected with ECL detection kit.

Determination of antioxidant activity

The levels of SOD, GSH-px, MPO and MDA were evaluated with assay kits from Nanjing Jiancheng Bio-engineering Institute (Jiangsu, China), following the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of IL-1β, TNF-α, IL-18 and IL-10, in the cell lysates were evaluated with ELISA kit following the manufacturer's protocols. The samples were added to wells and biotin-conjugated primary antibodies were plated into the wells as well before the addition of avidin-conjugated horseradish peroxidase (HRP). Then enzyme substrate was added for color development. The absorbance of the contents was measured with a microplate reader at a wavelength of 350 nm (R&D systems, Minneapolis, MN, USA).

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was conducted by Student's t-test using GraphPad. $P < 0.05$ was considered statistically significant.

RESULTS

CHE increased LPS-induced cell viability and reduced cell apoptosis

The viability of HFL1 cells was reduced when stimulated with LPS, but CHE treatment reversed it in LPS-stimulated cells ($p < 0.05$, Figure 1 A). Expressions of Bcl-2 and PARP were lowered, but Bax and cleaved-PARP were enhanced in LPS-induced cells. CHE treatment significantly reversed these alterations in a dose-dependent manner ($p < 0.05$, Figure 1 B). Moreover, LPS stimulation significantly induced changes in the ratio of apoptotic cells, while CHE reversed apoptosis in the cells ($p < 0.05$, Figure 2). Thus, CHE induced cell proliferation and reduced cell apoptosis in HFL1 cells.

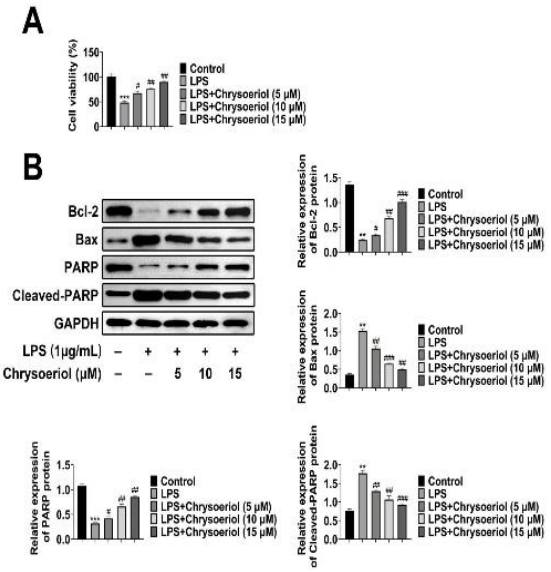

Figure 1: CHE increased LPS-induced cell viability and reduced cell apoptosis A. The cell viability in control, LPS, LPS + CHE (5 μM), LPS + CHE (10 μM) and LPS + CHE (15 μM) groups. B, The expression level of Bcl-2, bax, PARP, and cleaved-PARP in control, LPS, LPS + CHE (5 μM), LPS + CHE (10 μM) and LPS + CHE (15 μM) group. ***$P < 0.001$ vs control; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs LPS

CHE ambivalent oxidative stress in LPS-induced cells

Lipopolysaccharide (LPS) stimulation is believed to induce oxidative stress in cells. Therefore, the level of MDA and MPO were enhanced and SOD and GSH were reduced in LPS stimulated cells (Figure 3 A-D). The Chrysoeriol treatment significantly improved these parameters, indicating its effects in anti-oxidative stress.

CHE ameliorated cell inflammation in LPS-stimulated cells

To determine the inflammatory response in the LPS-induced cell model, the level of IL-10, IL-1b, IL-18 and TNF-a were measured in the cells, and the data revealed that LPS significantly induced the expressions of IL-1b, IL-18 and TNF-a, and increased the level of IL-10, (Figure 4 A - D). Chrysoeriol administration stimulated the inflammatory response as shown by reduction in IL-1β, IL-18 and TNF-a, and further enhanced IL-10 (Figure 4 A - D).

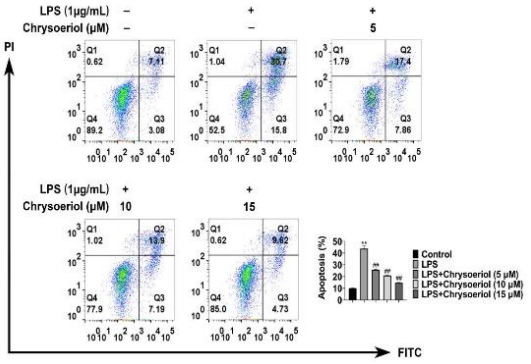

Figure 2: CHE inhibited LPS-induced activation of PI3K/AKT/mTOR signaling pathway in pneumonia cells. Cell apoptosis as detected via Flow cytometry in control, LPS, LPS+CHE (5 μM), LPS + CHE (10 μM) and LPS+CHE (15 μM) groups. **$P < 0.01$ vs control; ##$p < 0.01$ vs LPS.

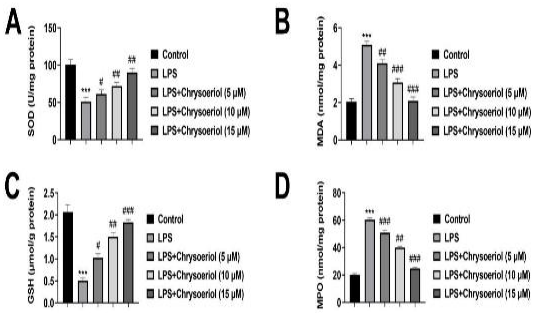

Figure 3: CHE improved oxidative stress in LPS induced cells. A-D The levels of SOD (A), MDA (B), GSH (C) and MPO (D) in control, LPS, LPS + CHE (5 μM), LPS + CHE (10 μM) and LPS + CHE (15 μM) groups. ***$P < 0.001$ vs control; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs LPS

CHE inhibited LPS-induced activation of PI3K/AKT/mTOR signaling pathway in pneumonia cells

To delineate the potential effects of CHE-mediated cell proliferation and apoptosis of LPS-treated pneumonia cells, the activation of PI3K/AKT/mTOR signaling was evaluated in

each group, and it was found that the expression levels of p-AKT, p-PI3K and p-mTOR were upregulated in LPS induced cells (Figure 5), and CHE treatment reversed the enhanced expression of p-AKT, p-PI3K and p-mTOR (Figure 5). The data indicate that CHE inhibited LPS-induced activation of PI3K/AKT/mTOR signaling pathway in HFH cells.

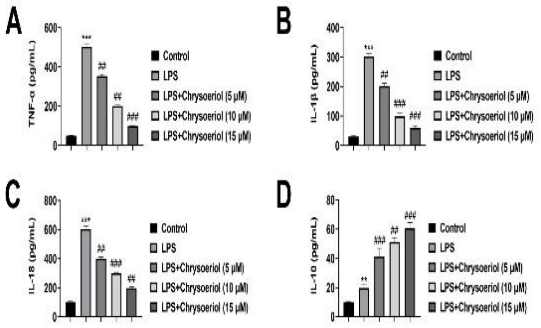

Figure 4: CHE ameliorates cell inflammation in LPS-stimulated cells. A-D, The level of TNF-a (A) IL-1b (B), IL-18 (C), and IL-10 (D) in control, LPS, LPS + CHE (5 μM), LPS + CHE (10 μM) and LPS + CHE (15 μM) groups. ***P < 0.001 vs control; #p < 0.05, ##p < 0.01, ###p < 0.001 vs LPS

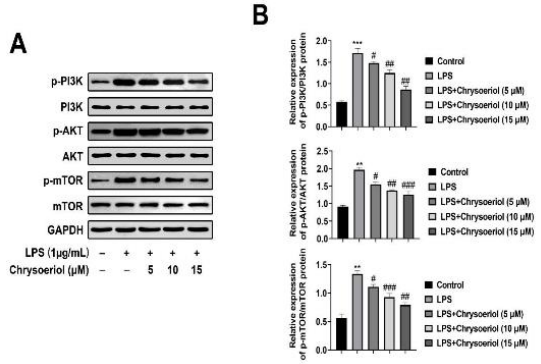

Figure 5: CHE inhibited LPS-induced activation of PI3K/AKT/mTOR signaling pathway in pneumonia cells. A and B: The levels of p-AKT, p-PI3K and p-mTOR in control, LPS, LPS+CHE (5 μM), LPS + CHE (10 μM) and LPS + CHE (15 μM) groups. ***P < 0.001 vs control; #p < 0.05, ##, p < 0.01, ###p < 0.001 vs LPS

DISCUSSION

Pediatric pneumonia is a lung inflammation caused by a variety of pathogens [11]. It is a common respiratory disease in children, and the main symptoms include fever, cough, shortness of breath, dyspnea [12]. Pneumonia is one of the most common illnesses in infants and children. The main treatment strategy consists of symptomatic and anti-infection treatments, as well as prevention of complications [13]. The incidence of severe pneumonia is high, and the existing antibacterial drugs rarely achieve good therapeutic effects due to the existence of drug resistance. Therefore, in order to further improve the therapeutic effects on patients, it is still necessary to develop new and more effective therapeutic drugs.

In this study, chrysoeriol increased cell proliferation, reduce cell apoptosis, regulate inflammatory progression, and inhibit activation of PI3K/AKT/mTOR signaling pathway in pneumonia cells. The data therefore confirms that it could serve as a promising drug for pneumonia treatment.

In the *in vitro* assays performed in the present study, the effects of chrysoeriol on cell viability, apoptosis, and inflammation of LPS-induced HFH cells were determined. Chrysoeriol suppressed the progression of pneumonia. Chrysoeriol has been reported to ameliorate the expression of COX-2 via NF-κB, AP-1 and MAPK in LPS-induced murine macrophages [9]. In addition, the antioxidative effects of chrysoeriol modulates mitochondrial function. Its anti-tumor effects have also been reported [8,14].

Chrysoeriol prevented TNFα-induced CYP19 expression via the downregulation of EGR-1 in breast cancer cells [10]. Importantly, chrysoeriol also has a variety of pharmacological properties such as anti-inflammatory, and antioxidant [15]. The results further confirmed its effects on inflammation. However, the precise mechanism needs further investigation.

Interestingly, honeysuckle can potentially prevent acute lung inflammation by decreasing the levels of pro-inflammatory factors in serum and bronchoalveolar lavage fluid, decreasing the levels of oxidative stress factors, and increasing the activities of SOD and GSH in lung tissues. Hence, its component, chrysoeriol, has an effect on inflammation of lung tissues and pneumonia [8,14,15]. Chrysoeriol has also been reported to suppress the inflammation of LPS-induced pneumonia cells [8,14.15].

Thus, in the present study, chrysoeriol reduced pneumonia inflammation by inhibiting PI3K/AKT/mTOR pathway. The effect of this pathway on the inflammation of pneumonia has been widely reported [16,17]. For example, IL-17A-producing T cells exacerbated fine particulate matter-induced lung inflammation by suppressing PI3K/Akt/mTOR-mediated autophagy [17]. Another study indicate that microRNA-4485 ameliorated severe pneumonia

via inhibition of the PI3K/AKT pathway [18]. These studies confirmed that PI3K/Akt/mTOR axis is a promising target for pneumonia treatment.

CONCLUSION

Chrysoeriol increases cell viability, suppresses apoptosis, and lowers oxidative stress in an *in vitro* cell model of LPS-induced pneumonia cells. Furthermore, it reduces LPS-induced pneumonia cell inflammation, as well as the activation of PI3K/AKT/mTOR axis. However, it is necessary to expand this study to include *in vivo* investigations.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yan Wang designed the experiments and carried them out. Qiuyue Hong analyzed and interpreted the data, prepared the manuscript with contributions from all co-authors.

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