Protective Effect of Rosmarinic Acid on Influenza Virus-induced Pneumonia in Mice

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Abstract Influenza virus induces pneumonia or flu in living body, and the progress is severely dependent on the host immune system, environmental factors and strain for infection. H1N1 Pneumonia progresses quickly in the living organism, leading to severe respiratory failure or refractory pneumonia, with greater mortality than bacterial pneumonia. Rosmarinic acid has known for its an anti-inflammatory and anti-viral effect. The current investigation was designed to scrutinize the protective effect of Rosmarinic acid on influenza virus-induced pneumonia in mice and explore the possible mechanism. The protective effect of Rosmarinic acid was assessed on influenza induced virus infection in mice. The survival rats, virus yield and mean survival time were also estimated. Pro-inflammatory cytokines such as interleukin-10 (IL-10), interferon-γ (INF-γ), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) were scrutinized in the serum and lung tissue. NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1 level also estimated. Rosmarinic acid considerably increased the survival rate, decreases the virus yields and prolongs survival., Rosmarinic acid significantly (P<0.001) up-regulated the INF-γ, IL-10 and down-regulated the level of IL-6 and TNF-α in the serum and lung tissue. Rosmarinic acid significantly reduced the expression of NLRP3, ASC and Caspase-1. Based on the obtained result, we can conclude that Rosmarinic acid exhibited a protective effect against the virus infection through inflammatory reaction.

Keywords: Pneumonia, Rosmarinic acid Inflammation, Cytokines


1. Introduction

Influenza induced the pneumonia or flu like illness, whose progress depends on several factors such as the immunity of the host immune, environmental factors and infecting strain. H1N1 Pneumonia, culminating rapidly in the respiratory system leading to severe respiratory failure or refractory pneumonia, with higher mortality rate than bacterial pneumonia [1,2]. H1N1 infection is a life-threatening disease. Influenza virus is highly infectious to respiratory system and causes acute respiratory disease that induces the higher rate of mortality and morbidity worldwide [3]. Previous research suggested that infection induced by the influenza virus to the people occurred from the ancient times [4,5]. Currently available treatments for influenza are neuraminidase inhibitors such as zanamivir and oseltamivir, but the mentioned treatment having limitation due to development of the resistance and serious side effects [6,7]. Due to development of the resistance by the current treatment line, urgent need to develop the effective drug to inhibit the viral infection along with minimal the side effects [7,8]. From the ancient time, traditional medicine system having great history to treat the viral infection [9]. Presently, the traditional medicinal plants are recognised attentively as effective source to develop as the antiviral drugs [9,10].

Current studies indicated that the innate immune system including the inflammasome has been shown to play a significant role in various diseases such as Alzheimer's disease, atherosclerosis, and type II diabetes mellitus [11,12]. NLRP3 is consist of pyrin domain containing 3 protein (NLRP3) inflammasome and nucleotide binding domain and leucine rich repeat containing gene (NLR), the adaptor protein contains the C-terminal caspase recruitment domain (ASC) and effector protein caspase-1 [11,12,13]. The inflammatory protein NLRP3 activated in response to a wide range of exogenous and endogenous stimuli such as infecting micro-organisms. Moreover, infecting micro-organism such as S. aureus-hemolysin was exhibited to induce the secretion of interleukin-1β (IL-1β) and activation of caspase-2 in the mouse and human monocytic cell via NLRP3 inflammasome [11,12,13]. Additionally, the activation of NLRP3 has been found to play a significant role in a serve S. aureus a-hemolysin model [11].

The Rosmarinic acid is an ester of 3,4-dihydroxyphenyllactic acid and caffeic acid present in over 240 species of plants. Its showed the numerous
pharmacological effects such as anti-viral, anti-inflammatory, anti-angiogenic and anti-tumoral effects [14,15]. Many literature showed that Rosmarinic acid showed the inhibitory effect against the UV exposure skin photocarcinogens is as photo-protective and also exhibited the preventive effect against the ultraviolet (UVB) induced deoxyribose nucleic acid (DNA) injury [16,17]. In addition, Rosmarinic acid suppressed the proliferation of cells through induction apoptosis in hepatic stellate cells. Rosmarinic acid also induced death of lymphoblastic leukemia cells through various cell death pathways [16,18]. In addition, few studies suggested that Rosmarinic acid has been effective in preventing eye diseases [16,17]. Due to anti-inflammatory and anti-viral properties of Rosmarinic acid, we used to treat the influenza virus induced pneumonia in mice in this experimental study, and explore the possible pathway.

![Structure of Rosmarinic Acid](image1)

**Figure 1.** Structure of Rosmarinic Acid

### 2. Material and Method

#### 2.1. Virus and Chemical

The cells (MDCK) were maintained in the Eagle’s minimum essential medium containing penicillin, streptomycin and calf serum. For the study, the Influenza virus received from the Institute of Virology, Wuhan University and seeded in the MDCK cells. Ribavirin (O2 Pharmaceutical, Zhejiang China), Rosmarinic acid was purchased from Sigma Aldrich, (U.S.A). All the reagent used in the current experimental study was of analytical grade and procured from the reputed vendor.

#### 2.2. Animal

BALB/c female mice (20-25 g; 6-8 wk old) were used for the current experimental protocol. The mice were received from the Laboratory of Animal Research Centre and kept in the standard laboratory condition (23±2°C) relative humidity (40-70%) and day/night (12/12 h) cycle. Reed and Muench method were used to induce the medium infectious dose in the mice. The whole experimental protocol was performed according to the Animal Care and Use Committee of Institute.

#### 2.3. Experimental Design

For the induction of pneumonia, the mice were anesthetized using the diethyl ether as previously explained dose. The mice were received the intranasally challenged with 30 µl of mouse adapted 10 × LD₃₀ (50% of lethal dose) IVA and after that the mice were divided into the group such as NC group, VC group, ribavirin received group and RA received group. All group contained the 10 mice. Ribavirin and RA were administered intrastracically for 5 days in the infected mice, although the NC and VC group mice received the saline at the same time intervals. The mice were physically scrutinized such as checking the weight or any death of the mice during the experimental duration [19,20].

### 2.4. Biochemical Analysis

For the biochemical analysis, the blood samples were collected through puncturing the retro orbital plexus and centrifuged the blood samples at 10,000 rpm for 15 min to obtain the serum and stored at -80°C for further studies.

### 2.5. Estimation of Cytokines

Tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-10 (IL-10) were estimated in the lung homogenate or serum via using the enzyme linked immunosorbent assay (ELISA) reagent kits (Boster Biotech. Inc., Wuhan, China). The whole cytokine estimation was performed according to the manufacture instructions. Briefly, all the samples were incubated in the 96 well plates along with the monoclonal antibody for 2 h at 37°C, followed by washed with the phosphate buffer saline (PBS). Subsequently, added the polyclonal antibody and again incubated for 2h and repeated this process 3 times. After that tetramethylbenzidine substrate mixed and incubated for ½ h and finally estimation done by taking the absorbance at 450 nm via using the microplate reader.

### 2.6. RT Quantitative (q)PCR

TRizol reagent was used for the isolation of total RNA from the from the lung tissues and finally removed the DNA from the RNA via using the RNase free DNase I via using the manufacture instruction. For RTqPCR, 1 µg (total RNA) was reverse transcribed to cDNA (1st strand) in 20 µl of mixture containing 4 µl (25 mM MgCl₂), 2µl reverse transcription buffer, 2 µl dNTP mixture, 0.5 µl recombinant RNase inhibitor, 15 µl AMV reverse transcriptase and 0.5 µg Oligo (dT) primers. The reaction conditions were as follows: 60 min (42°C) followed via 5 min (95°C) and finally qPCR was completed using the Sequence Detection System via using the SYBR Premix Ex Taq via using the manufacture instruction. GAPDH was used as the internal control. The PCR reaction system contained cDNA template (2 µg) dNTP, ligase, dNTP (200 µmol/l) buffer (10x buffer, each primer (20 pmol, Mg²⁺ (1.5 mmol/l)) and finally nuclelease free water. PCR was carried out as follows: 5 min (94°C) followed by 30 cycle (94°C).

### 2.7. Statistical Analysis

In the current experimental study, all the data were analysed statistically via applying the Dunnett's test. Statistical comparisons of data were performed using the ANOVA via using the Graphpad Prism 5 and P<0.05 was considered as the significant.
3. Result

3.1. Effect of Rosmarinic Acid on PR8 Viral in Lungs of Influenza Infected Mice

In the survival time trial, a range of dose of Rosmarinic acid was given in the experimental mice and concluded that lower dose had almost the same impact as higher dose. Figure 2 showed the effect of Rosmarinic acid on the viral load in lungs was scrutinized on different time intervals such as 2, 4 and 6 days. Figure 2 showed that the vehicle treated mice showed the increased virus yield and the its reached a peak (84.3×10^5 copies/ml) on day 4 and on the day it shows the viral load (65.3×10^5 copies/ml). As comparison with the virus control, Rosmarinic acid significantly decreased the virus yields on all days (59.5×10^5 copies/ml and 54.5×10^5 copies/ml) on day 4 and 6, respectively. Ribavirin also showed strong viral titers inhibition on all days.

3.2. Effect of Rosmarinic Acid on Pro-inflammatory Cytokines in Serum of Influenza Induced Mice

Figure 3 exhibited the effect of Rosmarinic acid on the antiviral cytokines level like IL-10 and INF-Υ; and pro-inflammatory cytokines including IL-6 and TNF-α in the serum of influenza virus infected mice. The mice in virus control group showed the increase level of antiviral cytokines such as IL-10, INF-Υ and pro-inflammatory cytokines viz., IL-6, TNF-α. Rosmarinic acid significantly up-regulated the antiviral cytokines such as IL-10, INF-Υ and down-regulated pro-inflammatory cytokines viz., IL-6, TNF-α on day 4 and 6.

3.3. Effect of Rosmarinic Acid on Pro-inflammatory Cytokines in Lung of Influenza Induced Mice

Figure 4 demonstrated the effect of Rosmarinic acid on the pro-inflammatory cytokines such as IL-6 and TNF-α; antiviral cytokines like IL-10 and INF-Υ. A similar momentum was observed in the cytokines level in lung tissue. Rosmarinic acid significantly reduced the pro-inflammatory cytokines such as IL-6 and TNF-α and enhanced antiviral cytokines like IL-10 and INF-Υ on the comparison the virus control on day 4 and 6.

Figure 2. exhibited the effect of rosmarinic acid on the mortality rate of mice infected with the S. aureus strains. *P<0.05, **P<0.01 vs S. aureus strains infected mice after treatment with PBS.
Figure 4. demonstrated the effect of rosmarinic acid on the pro-inflammatory cytokines level in the lung tissue of S. aureus infected mice. a: INF-γ, b: TNF-α, c: IL-6 and d: IL-10. Result was presented as mean ± standard deviation of three independent experiments. **P<0.01; ***P<0.001 vs. mice infected with S. aureus after treatment with PBS.

Figure 5. showed the effect of rosmarinic acid on the expression of NLRP3 inflammasome in S. aureus infected mice. a: NLRP3, b: ASC and c: Caspase-1 mRNA. Result was presented as mean ± standard deviation of three independent experiments. **P<0.01; ***P<0.001 vs. mice infected with S. aureus after treatment with PBS.

3.4. Effect of Rosmarinic Acid on NLRP3 Inflammasome

Figure 5 exhibited the effect of rosmarinic acid on the NLRP3, ASC and Caspase-1 expression. Virus control showed the increased expression of NLRP3, ASC and Caspase-1 and Rosmarinic acid significantly decreased the expression of NLRP3, ASC and Caspase-1.
4. Discussion

As far as we know, the main focus of the current study was to demonstrate that Rosmarinic acid exerts a beneficial effect against the H1N1 induced pneumonia in the mice and its effect with the down-regulation of inflammatory reaction along with reduction of NLRP3 inflammasome. There was growing evidences that some natural products protected mice from pneumonia. It is well proved that the Rosmarinic acid had antimicrobial, antibacterial effect against the various bacterial strains [16,17].

Previous studies suggested that the influenza induced the sporadic and pandemics which prove that influenza is a major health burden [21]. The quick expansion of viral pneumonia induced via aggressive inflammation leading to high mortality and morbidity, demonstrating the significance of exploring successful approaches to boosting the viral pneumonia during H1N1 infection [22,23]. In the current experimental protocol, we used the oral administration of Rosmarinic acid against the PR8 induced infection in mice and explored the protective effect. In the study, we found that the Rosmarinic acid increased the survival rate and also exhibited the prolonged mean survival time in the PR8 induced infection in mice. It is well documented that down-regulation in virus titer is the significant for decreasing the mortality due to influenza virus infection. It was observed that the Rosmarinic acid able to reduce the viral load in the lungs at different time interval, suggesting that the viral infection induced reduction via Rosmarinic acid treatment.

Influenza-induced pneumonia is responsible for a strong inflammatory response from the host, which induces rapid and excessive infiltration of inflammatory cells [24]. Pro-inflammatory cytokines such as IFN-γ inflammatory cytokines and Th-1 pro-inflammatory cytokines, IL-10 as Th2 cytokines and TNF-α and IL-6 as pro-inflammatory cytokines were shown to be generated in response to rodent model influenza virus infection [19,20]. Such cytokines can alter the course of infection, and play an important role in pathogenesis triggered by the influenza virus [19,20]. In the current experimental study, we observed that the pro-inflammatory cytokines such as INF-γ, TNF-α, IL-6 and IL-10 in the lung tissue and the serum in the all experimented group mice. Rosmarinic acid altered the level of cytokines and provided the therapeutic effect.

IFN-γ is an immune-regulatory cytokines, played a crucial role in the limiting the expansion of viral infection induced by influenza [25,26]. Previous literature suggested that the administration of IFN-γ protected the mice from the influenza infection at early stage of infection [25,27]. The concentration of IFN-γ was observed high in the lung tissue and serum of influenza induced pneumonia in mice [25,28]. A similar result was found in our experimental group mice and administration of Rosmarinic acid reduced the increase level of IFN-γ and suggesting the protective effect on the influenza induced pneumonia. Additionally, IL-10 is known as an anti-inflammatory cytokine able to control immune responses [25,26]. Preclusion of IL-10 in-vivo during influenza infection led to increased pulmonary inflammation and lethal injury [29,30]. In the current experimental study, we observed the increased level of IL-10 in the influenza induced pneumonia in mice and rosmarinic acid reduced the level of IL-10 and suggesting the inflammatory effect on the influenza induced pneumonia.

Pro-inflammatory cytokines such as IL-6 and TNF-α are consider as the important role in the acute inflammation reaction of infection and tissue repair [31,32]. Increase level of IL-6 and TNF-α after viral infection can cause pneumonia, anorexia and viremia, and this activity may contribute to lethal injury [33,34]. Previous study suggested that the reduction of TNF-α could suppress the severity of virus specific lung immunopathology and the level of IL-6 also use as the indicator to estimate the progression of influenza. During the influenza induced pneumonia, the level of cytokine such as IL-6 and TNF-α considerably boosted due to increase the inflammatory reaction [29,35]. In the current experimental study, influenza induced pneumonia showed the increased level of IL-6 and TNF-α in the serum and lung tissue and rosmarinic acid significantly reduced the level of IL-6 and TNF-α in both serum and lung tissue.

Previous studies suggested that the NLRP3 inflammasome have a significant role in the host immune response to influenza and may be a significant target pathway to treat the influenza induced pneumonia [11,12,13]. In the current experimental study, we have found that rosmarinic acid reduced the ASC, caspase-1 and NLRP3 inflammasome mRNA expression in the lung tissue of experimental mice. It is well proved that the activation of NLRP3 inflammasome boost the activation and cleavage of caspase-1, which further increase the level of pro-inflammatory cytokines, resulting boost the inflammation [11,12,13]. In the current experimental study pro-inflammatory cytokines secreted from the lung tissue in the influenza induced pneumonia mice. Collectively, result suggested that the rosmarinic acid exerted a beneficial effect against the influenza induced pneumonia, probably via reduction of NLRP3 inflammasome.

5. Conclusion

Collectively, we can say that Rosmarinic acid inhibited the influenza induced pneumonia in the mice. Rosmarinic acid suppressed the inflammatory response in the influenza induced pneumonia in the mice. Rosmarinic acid reduced the expression of ASC, caspase-1 and NLRP3 inflammasome and suggested the preventive effect against the influenza induced pneumonia via down-regulated the inflammatory reaction. Furthermore, more experimental study needed to clarify the possible mechanism by which rosmarinic acid suppress the NLRP3 inflammasome activation.

Abbreviation

IL-1β- Interleukin-1β, IL-10- Interleukin-10, IFN-γ-Interferon-γ, IL-6=Interleukin-6, TNF-α- Tumor necrosis factor-α, NLRP3- NOD-, LRR- and pyrin domain-containing protein 3, ASC- Adaptor apoptosis-associated speck-like protein containing a CARD, IL-1β-Interleukin-1β, UVB- Ultraviolet, DNA- Deoxyribose nucleic acid, RA- Rosmarinic acid.
References


