The effect of carvacrol on respiratory syncytial virus infection in mice model: caution in the use of herbal medicines

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ABSTRACT

Background and Objectives: Respiratory syncytial virus (RSV) is one of the most common viruses associated with acute lower respiratorytract infections in infants, young children, and the elderly. Due to a lack of effective anti-viral drugs or vaccines, using an immunomodulatory strategy is probably the best option to decrease the burden of RSV disease. Here, we studied carvacrol as a known immunomodulator on RSV infection outcome in a mice model.

Materials and Methods: Balb/c mice were infected by intranasal inoculation of RSV-A2, and treatment started daily 24 h after infection. Mice were sacrificed on day five after infection and experimental analyses were performed to study airway immune cell influx, CD4 and CD8 subtypes, cytokine/chemokine secretion, lung histopathology, and viral load.

Results: Results showed that using carvacrol enhanced immune cell influx, cytokine/chemokine production, and virus titer, and aggravated lung pathology. Our result showed that carvacrol administration increased viral titer compared to the RSV-PBS group. Also, carvacrol significantly induced IFN-γ production and did not induce IL-10 production. Besides, carvacrol non-significantly increased lymphocytes and monocytes count but did not affect the neutrophil count.

Conclusion: Carvacrol at the concentration of 80 (mg/kg) did not show immunomodulatory activity to alleviate the RSV infection outcome. Further research is needed to uncover the effects of the carvacrol intervention on virus replication and immune responses following RSV infection. Many herbal remedies in use contain carvacrol. However, the use of herbal remedies to treat viral respiratory infections such as RSV has to be performed with caution.

Keywords: Human respiratory syncytial virus; Carvacrol; Immunomodulation; Interferon-gamma; Interleukin-10

INTRODUCTION

Respiratory syncytial virus (RSV) is the most common cause of respiratory illness in infants, young children and, the elderly, worldwide (1). Disease symptoms vary from a mild upper respiratory tract infection to severe bronchiolitis and pneumonia requiring hospitalization (2). The virus accounts for about 50% to 90% of hospitalizations for bronchiolitis and up to 40% of pneumonia (3). RSV disease mortality rates in children aged <5 years is estimated to be 200,000, with 99% occurring in developing countries (4). There is growing evidence that early life severe RSV infection may lead to recurrent wheezing and the development of asthma in later life (5). Due to a lack of vaccines, an effective treatment strategy is needed to decrease the burden of disease and its considerable long-term effects (6).

Although strategies that solely targeted the virus (anti-viral drugs) are obvious candidates to treat...
RSV illnesses, they did not meet their expectations and remain in infancy (7). The lung pathology developed in RSV-infected individuals is characterized by strong pulmonary inflammation, which is known as immunopathology (8). Inflammation in the upper and lower airways of infants with acute bronchiolitis is dominated by an intense immune cell influx, and this is accompanied by pro-inflammatory and anti-inflammatory chemokine/ cytokine production and disease severity (2). There is, however, increasing evidence to suggest that targeting of RSV-associated immune response can alter virus replication, inflammation, and a pathology leading to improved disease outcomes (7, 9).

There are many options as immunomodulatory compounds, and the tendency to use herbal compounds will increase due to their low price and high efficacy (10). Carvacrol, as an herbal compound found in many plants of the Lamiaceae family, has immunomodulatory and anti-inflammatory action. It also has many other biological effects such as anti-obesity, anti-fungal, anti-viral, anti-bacterial, neuroprotective, cardioprotective, and anti-oxidant properties (11). Although the mechanisms of immunomodulatory and anti-inflammatory action of carvacrol are not clear completely (12), it has been discovered that carvacrol reduces the recruitment of immune cells, especially neutrophils (13). Furthermore, it has been shown that it increases IL-10 production and the ratio of IFN-γ/IL-4 as well as Th1/Th2 (12, 14). Here, we studied carvacrol as an herbal immunomodulator on RSV infection outcome in a mouse model.

MATERIALS AND METHODS

Twenty-four female Balb/c mice (6-7 weeks old, weighing 17-20 g) were purchased from the Institute Pasteur of Iran (Karaj, Iran). Animals were kept in-house for one week before the experiment in the animal house of Tehran University of Medical Sciences with access to standard laboratory food and water ad libitum. This study was approved by the animal ethics committee of the Tehran University of Medical Sciences (No. 1398.047). Carvacrol was obtained from Sigma-Aldrich with a purity of 98% and diluted with normal saline serum as recommended. Virus stock was prepared as described previously by our groups (15).

Mice were randomly assigned into four groups: PBS-PBS, PBS-CAR, RSV-PBS, and RSV-CAR. According to the mentioned groups, mice were anesthetized with ketamine/xylazine and intranasally infected with RSV-A2 at 7.8 × 10⁴ pfu/50 μl/mice dose. The carvacrol was injected i.p. 24 h after viral infection once a day at a dose of 80 (mg/kg). The control groups received an equal volume of phosphate-buffered saline (PBS). Animals were sacrificed by a lethal dose of ketamine (i.p.) on day five after infection, and bronchoalveolar lavage fluid (BALF) and lungs were obtained to measure airway immune cells influx, CD4⁺, and CD8⁺ T-cell populations, cytokine/chemokine secretion, lung histopathology, and viral load.

The lung was lavaged through a catheter inserted into the trachea and by flushing with ice-cold PBS, according to our previous work (15). The obtained BALFs were centrifuged, and the total number of cells was counted with Neubauer chambers. Differential leukocyte counts were performed on smears stained with Giemsa dye using standard morphological criteria. The amount of IFN-γ and IL-10 cytokines were determined in the BALF supernatants by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (R&D Systems and Invitrogen, respectively).

The CD4⁺ and CD8⁺ T-cell population in BALF was measured with the flow cytometry method. The specific volume of each sample that consisted of 2 × 10⁶ cells was washed twice with FACS buffer (2% BSA in PBS) and incubated with fluorochrome-conjugated anti-CD3, anti-CD4 and anti-CD8 immunoglobulins (BD bioscience, USA) for 45 min at 4°C in the dark. The cells were washed twice with FACS wash buffer and analyzed with a flow cytometer (Life technologies, USA). FlowJo® software (Tree Star, Inc., Ashland, OR, USA) was used to analyze the data.

Histology slides were prepared as described previously by our group (15). Prepared slides were evaluated by light microscope, and lung pathology, peribronchial and perivascular infiltration in the lungs were scored as described previously (16). The average of the sum of each reading was compared among groups.

Total RNA was extracted from supernatants of BALF using viral high pure nucleic acid extraction kit following manufacturers’ instructions (Roche, Germany). Real-time RT-PCR was carried out on an ABI PRISM 7900 sequence-detection system (Applied Biosystems, USA) using TaqMan PCR Master Mix (Primer Design, UK), and results were expressed as copy number/ml as described previously (15).
RESULTS

Our results showed that the carvacrol increased viral titer compared to the RSV-PBS group (Fig. 1). RSV infection stimulates an inflammatory response in the airways comprising cytokines production and, a mixed population of immune cell infiltration, resulting in lung pathology. Compared with the control group, carvacrol significantly induced IFN-γ production (p-value = 0.024) and did not induce IL-10 production (Fig. 2). In this experiment, the level of immune cell infiltration was enhanced following carvacrol treatment in RSV-infected mice (RSV-CAR group), although it was not statistically significant compared to the RSV-PBS group (Fig. 3). The differential analysis of the leukocytes in the BALF of mice showed that carvacrol administration non-significantly increased lymphocytes and monocytes count, but did not affect neutrophil count (Fig. 4). Carvacrol treatment increased CD4+ and CD8+ lymphocytes count, but CD4/CD8 ratio non-significantly decreased following carvacrol treatment in RSV-infected mice (RSV-CAR group) (Fig. 5). According to the immune responses and viral titer, lung pathology scores were higher in mice of the RSV-CAR group than the RSV-PBS members (Fig. 6). In this experiment, exposure of mice to the PBS or carvacrol alone did not result in differences within the control groups.

DISCUSSION

The immunomodulatory activity of carvacrol has been well investigated in several studies (11, 17). However, the results of this study showed that carvacrol not only did not alleviate the RSV immunopathology but also worsen the infection outcome. Using...
carvacrol enhanced the virus titer following by an increased cytokine/chemokine production and immune cells influx and ultimately aggravated lung pathology. The numbers of neutrophils did not change due to carvacrol administration, which was against our presumption that carvacrol could decrease the infiltration of neutrophils. Furthermore, flow cytometry results displayed an increase in CD8 and CD4 absolute number and a decrease in CD4/CD8 ratio, which indicates the exaggeration of immune responses with changing toward, Th1 direction. The higher score of histopathology of lung slides in the RSV-CAR group delighted the more severe infection resulting from of carvacrol administration.

The results were totally against our expectation, and we assumed some hypotheses to explain the promiscuous effect of carvacrol in the RSV mice model. It is evident that the concentration of agents is very critical in the experiment and can even distort the results. As an example, Rajabli et al. showed that carvacrol at the concentration of 1 mM increased NheA and HblC toxins in Bacillus cereus infection, but at the concentration of 2 mM and 8 mM inhibited their production (18). In Stojanovic et al. study, carvacrol at a concentration of 10 mg/kg caused a decrease in α-amylase and lipase activities that led to the amelioration of pancreatic damage in a rat model, but at a concentration of 50 and 100 (mg/kg) increased the activities of the enzymes, and exacerbated the condition (19). Although the administrated dose of carvacol...
control (80 mg/kg) in this study was chosen according to the literature (13), investigating another dose could be helpful.

One of the critical findings in the results of this study is the increase in the virus titer following carvacrol administration. Therefore, the finding of its possible reasons might be helpful in future studies. An assumption could be that the alternation of the cells' situations resulting from carvacrol administration made a better environment for viruses to infect cells and replicate in them however; its mechanism is unknown. Another reason could be that because of the immunomodulatory effect of carvacrol, it can reduce immune responses against RSV and permitted viruses to replicate better and infect more susceptible cells in the early days of administration. Therefore, the reduction of the inflammatory cytokines/chemokines might delay effective responses against RSV in the early stage of infection. And because of the increase in viral load, the production of inflammatory cytokines/chemokines continues in the rest days of infection that would worsen the condition. Thus, the most appropriate time to apply carvacrol might be several days after RSV infection.

The effects of Immunomodulatory agents are like a double-edge sword in viral infections. Appropriate timing and duration play a critical role (20). In some cases applying an immunomodulatory agent like corticosteroid did not have any positive effects on the outcome of different respiratory infections (21).

Although, notably, the dose of carvacrol and the time of its applying are crucial, its effects could be various in the different study contexts. For example, while Mahmoudi et al. showed that carvacrol at the concentration of 5 and 10 (mg/kg) led to a decrease of IFN-γ and increase of TGF-β levels in the experimental autoimmune encephalomyelitis mice (22), Kianmehr et al. implicated the opposite effects, in which the level of IFN-γ increased, however, TGF-β decreased in mice that were sensitized by ovalbumin and treated with 75, 150, and 300 μg/mL concentrations of carvacrol (14). Silva Lima et al. indicated that carvacrol has no effects on the TNF-α level in the treated mice at a concentration of 50 and 100 mg/kg (12), but the study on the mice that faced the carrageenan-induced hyper nociceptions, showed that carvacrol caused the decrease of TNF-α level (23).

CONCLUSION

In conclusion, carvacrol at the concentration of 80 (mg/kg) did not show immunomodulatory activity to alleviate the RSV infection outcome. Further research is needed to uncover the effects of the carvacrol intervention on virus replication and immune responses following RSV infection. Many herbal remedies in use contain carvacrol. However, other active substances are included too (24), but the use of herbal remedies to treat viral respiratory infections such as RSV and COVID-19 have to be performed with caution (25).

Also, due to the SARS-COV2 pandemic and the consideration of herbal remedies (25, 26), the results of this study can be a warning in this regard so that the misuse or overuse of such herbal remedies may increase inflammation and disease outcomes in people contrary to the expected effects. Therefore, caution should be taken to use such herbal medicines so that the amount and timing of their use and the target tissue are of particular importance.

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