β-Caryophyllene ameliorates the Mycoplasmal pneumonia through the inhibition of NF-κB signal transduction in mice

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A B S T R A C T

Background: Pneumonia is a frequent infectious disease that mainly affects the children and the global death rate is nearly 19% among children at the below 5 age. β-caryophyllene is an active compound, mainly occurs in the spices and it possesses immense biological activities.

Objective: This investigation deliberated to scrutinize the beneficial actions of β-caryophyllene against the M. pneumoniae induced pneumonia.

Methods: The pneumonia was stimulated to the BALB/c mice by infecting them with 100 μl of M. pneumoniae for 2 days via nasal drops with the concomitant treatment with 20 mg/kg of β-caryophyllene. The total cells in the BALF of test mice were counted by using the Neuber chamber. The total protein and the pro-inflammatory cytokines status were examined by using the commercial ELISA kits. The PCR technique was used to measure the M. pneumoniae bacterial load. The NF-κB expression was investigated using western blotting. The lung tissues were analyzed microscopically.

Results: The β-caryophyllene notably diminished the total protein status, total cell count, and bacterial load in the pneumonia provoked mice. The marked reduction in the status of pro-inflammatory regulators was seen in the β-caryophyllene supplemented pneumonia mice. β-caryophyllene also down-regulated the expression of NF-κB thereby reduced the lung inflammation and tissue damages as seen in the result of histological analysis.

Conclusion: These findings were confirmed the therapeutic potential of β-caryophyllene against the M. pneumoniae-activated pneumonia in animals.

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1. Introduction

Mycoplasma pneumonia is a frequent infectious ailment that mainly affects the children and it is a major one among the top three pediatric diseases globally (Zasowski et al., 2014). As per the global statistics done by WHO, pneumonia is responsible for nearly 19% of children’s mortality (Guo et al., 2015). Mycoplasma pneumoniae is an infectious bacterium, which is responsible for numerous respiratory ailments among the children as well as teenagers. It is believed that the M. pneumoniae infected pneumonia is a prime cause of the whole community-acquired pneumonia (CAP) (Kishaba 2016, Ferrer et al., 2016). In recent times, M. pneumoniae emerged as a more resistant to the antibiotics and results in augmented prevalence of pneumonia and makes the ailment difficult to treat. Besides, M. pneumoniae infection can produce serious difficulties e.g. pulmonary fibrosis and asthma, also the victims of pneumonia need intensive care (Wood et al., 2017). In the human body, M. pneumoniae normally possesses the tissue and organ specificity with the special localization on the mucosal layer of alimentary canal, urogenital and respiratory tracts, and the mammary glands. MP is usually regarded as a membrane-bound parasite, sometimes enter into the cells and become intracellular residents (Yavlovich et al., 2004).

The incidences of M. pneumoniae stimulated pneumonia is gradually augmented in recent times and it developed its own resistance towards the many antibiotics, and become hard to treat. The infection of M. pneumoniae can also stimulate asthma through...
distressing the respiratory epithelium and ends in pulmonary fibrosis (Balish, 2014, Shangguan et al., 2014). Moreover, the exact pathological progression of *M. pneumoniae* infected pneumonia remains unclear. The previous report stated that the numerous immune cells e.g. macrophages, lymphocytes, and neutrophils are occurred in the pneumonia lesions (Kurata et al., 2014). Additionally, the overproduction of host immune reactions against the *M. pneumoniae* infection and overproduction of IL-8, IL-6, IL-1β, and TNF-α is concerned as a crucial contributor of pathological progress of *M. pneumoniae* infected pneumonia (Wang et al., 2017).

The *M. pneumoniae* infection normally interferes with the host immune mechanism thereby activated the pro-inflammatory responses via stimulating the intracellular signaling pathways (Youn et al., 2014, Shimizu 2016). During the *M. pneumoniae* mediated inflammation, the induced cells like macrophages accrete the augmented status of cytokines, ROS, and prostaglandin E2 (Seya and Matsumoto, 2002). The excessive accretion of these regulators playing a foremost role in the elimination of invading pathogen, and these sequentially regulates the several immunopathological reactions in the annoyed respiratory system. There are some adaptive mechanisms in the host immune system to guard against the dysfunctional inflammation at the time of infection.

The NF-κB signaling cascade is a vital transcription factor that occurs in all cells in an inactive phase and it can be triggered in response to the various stimuli. Hence it is concerned as a first-line responder to the numerous endogenous and exogenous stimuli e.g. free radicals, cytokines, oxidative stress, ROS, and viral or bacterial infections (Gilmore 2006). Moreover, the NF-κB plays crucial functions in the regulation of immune reaction to infections (Meffert et al., 2003). *M. pneumoniae* infection triggers the NF-κB signal transduction and macrophages via toll-like receptors to release the immuno-modulatory agents and IL-8, IL-18, TNF-α, and IL-1β that can speed up the immune reaction and result in the pneumonia progression (Oishi et al., 2011). The pro-inflammatory regulators are the imperative factors during the *M. pneumoniae* activated inflammatory responses. Previous investigations highlighted that the expression patterns of IL-1β, TNF-α, IL-8, and IL-6 in the lung tissues were noticeably augmented in the *M. pneumoniae*-infected animals (Pang et al., 2011). Among these regulators, the IL-8 was regarded as the vital arbitrator between the *M. pneumoniae* and neutrophils. It was also stated that the cellular components of *M. pneumoniae* can trigger the release of IL-8 in the BALF through the NF-κB in a time-dependant mode (Chmura et al., 2008).

Corticosteroids are known to the first-line therapeutic agent that can down-regulate the cell mediated lung injury as seen in the *M. pneumoniae* infection (Tagliabue et al., 2008). The corticosteroids were utilized clinically to treat the *M. pneumoniae* pneumonia and employed to suppress the cellular inflammatory reactions. It was often reported with the numerous deleterious outcomes in the prolonged administration (Miyaehita et al., 2007). The extended ineffective treatments to the *M. pneumoniae* infection suppress the diffusion of gas in pulmonary region even after the retrieval from the *M. pneumoniae* stimulated pneumonia. The repeated incidences of *M. pneumoniae* infection can also destruct the integrity of airway structures in the affected children. Consequently, it was highly appreciable to develop the novel curative agent to heal the *M. pneumoniae* stimulated pneumonia and guard the respiratory system (Pereyre et al., 2016).

The β-caryophyllene (β-CP) is a bicyclic sesquiterpene copiously occurs in the cinnamon, pepper, oregano, and clover. It gained an immense research interest that owing to its safeness and dietary accessibility (Sharma et al., 2016, Gertsch et al., 2008). β-caryophyllene already demonstrated the excellent organo-protective effects against the drugs induced harmful effects and chemical toxicants to the liver, kidney, pancreas, intestines, and brain (Calleja et al., 2013, Horvath et al., 2012, Basha and Sankaranarayanan, 2015, Cho et al., 2007, Ojha et al., 2016). Furthermore, β-caryophyllene also exhibited strong chemosensitizing and anticancer properties (Hanusova et al., 2017, Fidy et al., 2016). The curative potential of β-caryophyllene still remained unknown. Therefore, in this current study, we deliberated to scrutinize the beneficial actions of β-caryophyllene against the *M. pneumoniae* induced pneumonia.

2. Materials and methods

2.1. Chemicals

β-caryophyllene, Hayflick medium and other chemicals were procured from Sigma-Aldrich, USA. The protein assay kit was acquired from the Merck Millipore, USA. The ELISA assay kits for the estimation of pro-inflammatory cytokines were attained from the Raybiotech, USA. The western blotting kit was purchased from the Biorad, USA. Whole additional chemicals were bought from the Hi-Media, USA in diagnostic grade.

2.2. Collection and maintenance of *M. pneumoniae*

The *M. pneumoniae* culture was collected from ATCC, USA. The collected strain was maintained in the Hayflick medium that contains the PPLO broth, horse serum (25%), penicillin-G with yeast extract, tallium acetate (0.025%), glucose (0.5%) and phenol red (0.002%) until the investigation.

2.3. Experimental animals

The 4 weeks aged BALB/c mice, weighing about 23 ± 4 g were procured from the Institutional animal house and maintained in the hygienic cages on the usual laboratory environments (temperature 26 ± 1°C, air humidity 60 to 70%, and 12 h of light/dark series. Animals were sustained to the laboratory situations for 7-days before the investigation and during that time administered with the commercial pellet diet.

2.4. Experimentation and treatment procedures

Animals were estranged arbitrarily into four groups along with six animals in each. The group-I mice were served as the control without challenges. Group-II animals were infected with the 100 μl of *M. pneumoniae* for 2 days through the nasal drops to provoke the pneumonia. Group-III animals were administered with the 20 mg/kg of β-caryophyllene for 3-days simultaneously to the *M. pneumoniae* infection. Group-IV mice administered with the standard drug 100 mg/kg of Azithromycin for 3-days. After the completion of investigational time, all mice were anesthetized then cervical dislocation procedure was executed to sacrifice the animals.

2.5. Broncho alveolar lavage fluid (BALF) collection and the total cell count

The aliquots of 30 ml of buffered saline were instilled to the right middle lobe of each mouse for 5–6 times to gather the BALF sample. Then the collected BALF samples were centrifuged instantly at 6000 rpm for 7mins. Then the cell-free supernatant was taken in the clean tubes for additional investigations. The gathered BALF was observed under the microscope to count the total cells. Briefly, each BALF sample was placed on the Neuber chamber and the total cells were counted by using the microscope.
2.6. Measurement of total protein status

The level of epithelial injury and lung tissue permeability, the protein content in the BALF from each mouse was examined. The commercially procured kit was employed to scrutinize the protein content in the BALF of investigational animals as per the guidelines mentioned by the manufacturer (Merck Millipore, USA).

2.7. Detection of inflammatory markers level

The collected BALF from the investigational animals were subjected to centrifugation at 7000 rpm for 15 min to eradicate the debris. The cells free supernatant (diluted at 2-fold with the assay diluent) was utilized to detect the IL-6, IL-8, IL-1, and TGF with the help of the commercially procured ELISA assay kits by using manufacturer protocols (Raybiotech, GA, USA).

2.8. Measurement of M. pneumoniae load by PCR

The collected lung tissues were homogenized and then reconstituted with the DNA extraction buffer and the heated for 10 mins. After that, the suspension was centrifuged at 13000 rpm for 6 mins at 4 °C. The resulted supernatant was utilized as a PCR template. The primers for 16rRNA are sense 5'-GAATCAAGGTGAAAGGCCTGC-3' and antisense 5'-CTCTAGCCAT TACCTGCTAAAGTC-3' was utilized.

2.9. Western blotting analysis

The collected lung tissues were processed with the buffered saline and then homogenate by using the lysis buffer with protease inhibitors (0.01%). The tissue homogenate was centrifuged at 12,000 rpm at 4 °C for 6 mins then the supernatant was subjected to the SDS-PAGE (10%) separation. Then the samples were transferred to the PVDF membrane through electrophoresis. Later than, membranes blocked with fat-free milk (5%) and then incubated with the relevant primary antibodies at 4 °C. Then membranes again incubated with the secondary antibodies. The expression levels were scrutinized via the enhanced chemiluminescence kit (Biorad, USA).

2.10. Analysis of lung histopathology

The gathered lung tissues from both control and treated mice were cleansed with the addition of 5% of formaldehyde. Then the tissues were entrenched with paraffin wax and then the sliced into the slices (5 µm). Then tissues were stained by H&E. M. pneumonia induced tissue damages and other histological alterations were investigated with the help of an optical microscope.

2.11. Statistical assessment

Data were examined statistically by using the SPSS tool. The statistical variations between groups were scrutinized by one way ANOVA and then DMRT study. Data were portrayed as mean ± SD of triplicate values. p < 0.05 was regarded as significant.

3. Results

3.1. Effect of β-caryophyllene treatment on the total protein level in the BALF of pneumonia induced mice

The curative effect of β-caryophyllene against the MP infection stimulated epithelial damage and permeability of lungs was scrutinized through measuring the protein in the BALF and the results were illustrated in Fig. 1. The total protein level was elevated severely in the BALF of M. pneumoniae-activated animals. Interestingly, the administration of 20 mg/kg of β-caryophyllene to the M. pneumoniae triggered animals revealed the appreciably suppressed protein status (p < 0.05) in the BALF. It reveals that the β-caryophyllene was inhibited the M. pneumoniae infection stimulated lung permeability and epithelial injury. The outcomes of β-caryophyllene and the standard drug Azithromycin administered mice were parallel with each other.

Fig. 2 displayed that the total cells in the BALF of pneumonia provoked mice was dangerously enhanced than the control. The significant (p < 0.05) reduction in the total cell count was noticed in the 20 mg/kg of β-caryophyllene supplemented pneumonia mice. This result was revealed that the β-caryophyllene can ameliorate the M. pneumoniae induced increase in the inflammatory cells. The treatment with the Azithromycin to pneumonia stimulated animals were also displayed the notable diminution (p < 0.05) in the total cell counts n the BALF. The β-caryophyllene and azithromycin administered animals were demonstrated similar outcomes.

3.2. Effect of β-caryophyllene treatment on the total cells in the BALF of pneumonia induced mice

The M. pneumoniae bacterial load in the lung tissues of both control and β-caryophyllene supplemented pneumonia mice were investigated by the PCR technique and the result was illustrated in the Fig. 3. The PCR analysis result revealed that the M. pneumoniae bacterial load was severely augmented in the lung tissues of M. pneumoniae provoked mice, which in contrast to the control. Attractively, the 20 mg/kg of β-caryophyllene supplementation to the M. pneumoniae triggered animals demonstrated the notable (p < 0.05) suppression in the total cell load of M. pneumoniae in the lungs. This result proved that the supplementation of β-caryophyllene able to prevent the gathering of M. pneumoniae in the lungs thereby inhibits the M. pneumoniae growth. The β-caryophyllene and azithromycin administered animals were displayed the analogous outcomes.

3.3. Effect of β-caryophyllene on the M. pneumoniae bacterial load in the lung tissues of pneumonia induced mice

The M. pneumoniae bacterial load in the lung tissues of both control and β-caryophyllene supplemented pneumonia mice were investigated by the PCR technique and the result was illustrated in the Fig. 3. The PCR analysis result revealed that the M. pneumoniae bacterial load was severely augmented in the lung tissues of M. pneumoniae provoked mice, which in contrast to the control. Attractively, the 20 mg/kg of β-caryophyllene supplementation to the M. pneumoniae triggered animals demonstrated the notable (p < 0.05) suppression in the total cell load of M. pneumoniae in the lungs. This result proved that the supplementation of β-caryophyllene able to prevent the gathering of M. pneumoniae in the lungs thereby inhibits the M. pneumoniae growth. The β-caryophyllene and azithromycin administered animals were displayed the analogous outcomes.

![Fig. 1. Effect of β-caryophyllene on the total protein level in the BALF of pneumonia induced mice](image-url)

**Table 1.** Effect of β-caryophyllene on the total protein level in the BALF of pneumonia induced mice Values presented as a mean ± SD of triplicates (n = 6). Significance was determined by using one-way ANOVA followed by DMRT analysis. Note: ** indicates p < 0.05 compared with control and *** indicates p < 0.05 compared with pneumonia induced group.
3.4. Effect of β-caryophyllene on the inflammatory cytokine levels in the BALF pneumonia induced mice

The alleviating effects of β-caryophyllene against the M. pneumoniae infection triggered overproduction of IL-6, IL-8, IL-1, and TNF-α in the BALF were examined and the result was depicted in Fig. 4. The status of IL-6, IL-8, IL-1, and TNF-α was heightened severely in the BALF of M. pneumoniae provoked animals than control. However, the treatment with the 20 mg/kg of β-caryophyllene to the M. pneumoniae activated pneumonia animals demonstrated the noticeable (p < 0.05) lessening in the status of IL-6, IL-8, IL-1, and TNF-α on BALF. This data evidenced the anti-inflammatory capacity of β-caryophyllene against the M. pneumoniae infection triggered inflammation in mice. The outcomes of control, β-caryophyllene, and Azithromycin were uniformly similar.

3.5. Effect of β-caryophyllene treatment on the histopathological analysis of lung tissues of pneumonia induced mice

The M. pneumoniae infection stimulated tissue damages, inflammatory conditions and other histological alterations were inspected microscopically by using H&E staining. The histological investigation of lung tissues of M. pneumoniae challenged pneumonia mice were demonstrated the severe tissue injuries, vasodilation, congestion, and inflammatory cell penetrations while comparing it to the control (Fig. 5). The supplementation of β-caryophyllene was revealed the appreciable protection to lung tissues against the M. pneumoniae infection mediated injuries (Fig. 5). The 20 mg/kg of β-caryophyllene administration to the pneumonia activated animals were exhibited the usual tissue architecture, reduced inflammation, and the diminished inflammatory cell penetrations, which is contrast to the pneumonia stimulated mice. β-caryophyllene noticeably prevented the lung tissues of animals from the M. pneumoniae infection regulated damages.

3.6. Effect of β-caryophyllene treatment on the expression levels of NF-κB in the BALF pneumonia induced mice

Fig. 6 illustrating the inhibitory potential β-caryophyllene against the expression of NF-κB. Severely augmented status of NF-κB expression was noticed in the lung tissues of M. pneumoniae activated pneumonia mice, which in contrast to the control. The expression patterns of NF-κB were noticeably (p < 0.05) down-regulated by the 20 mg/kg of β-caryophyllene supplementation in the lung tissues of pneumonia mice. This outcome revealed that the β-caryophyllene was inhibited the NF-κB signaling cascade in the lung tissues of pneumonia provoked animals. The β-caryophyllene and azithromycin administered mice revealed the similar outcomes (Fig. 6).

4. Discussion

The Mycoplasma pneumoniae infection is a recurrent cause of pneumonia that primarily affects the children and adolescents. The global incidences of pneumonia are severely augmented nowadays and it becomes a life-threatening disease (Izumikawa 2016). The data from both pre-clinical and experimental works were highlighted that the cell-mediated immunity implicated by the host is closely linked with the M. pneumoniae infected pneumonia (Zhang et al., 2016). The M. pneumoniae infected pneumonia still remains under-diagnosed due to the lack of diagnostic procedures and other kinds of infections that either co-exist or mimics the M. pneumoniae. Moreover, the evidence from previous study denotes that the incidences of pneumonia are higher in the below 5 age children and the real impact on the elders and public health remains under estimated (Waites et al., 2017, Miyashita et al., 2008).

The cytoadherence of the pathogens is the imperative event during the infection that can lead to the restricted ciliary movement, cellular vasodilatation, loss of respiratory epithelial cellular integrity, and even cell death, which is directly relevant to the M. pneumoniae provoked pneumonia. The PCR technique is the most recurrently executed approach to detect the M. pneumoniae bacterial load. In this current exploration, the animals challenged with M. pneumoniae via intra-nasal route for the experimentation. Then the lung tissues were gathered and then homogenized, the supernatant was investigated via the PCR analysis. The results revealed the massive occurrence of M. pneumoniae bacteria in the lung tissues of pneumonia provoked animals than the control. This outcome denotes that the M. pneumoniae strain has a potent adhering capacity to the respiratory tracts the can cause severe injuries. Interestingly, the supplementation of 20 mg/kg of β-caryophyllene to pneumonia stimulated mice demonstrated a notable reduction in the M. pneumoniae bacterial load thereby prevented the airway and lung injuries from the M. pneumoniae infection (Fig. 3).

The TNF-α, IL-1β, IL-6, TGF-β1, and IL-8 are playing an imperative function in the regulation of inflammatory mechanisms. The IL-8 can induce the neutrophils, eosinophils, and T-lymphocytes thereby support the neutrophils degranulation, elastase release, and endothelial cell damages. The TNF-α is primarily generated by the macrophages and monocytes and it contributes to the severe inflammation-mediated lung damage with serious pneumonia (Ding et al., 2018). The TNF-α and IL-1β are often implicated in the early stages of inflammation moreover, they can trigger the lymphocytes and neutrophils accretion, enhance the vascular endothelial functions, and mediate the angiogenesis.
Fig. 4. Effect of β-caryophyllene on the levels of pro-inflammatory cytokines in the pneumonia induced mice. Values presented as a mean ± SD of triplicates (n = 6). Significance was determined by using one-way ANOVA followed by DMRT analysis. Note: ‘*’ indicates p < 0.05 compared with control and ‘**’ indicates p < 0.05 compared with pneumonia induced group.

Fig. 5. Effect of β-caryophyllene on the lung histopathology of the pneumonia induced mice. The normal tissue arrangements without any damages were seen in the control (Group-I). The lung tissues of pneumonia induced animals were demonstrated the severe inflammatory conditions, inflammatory cells infiltration, and tissue injuries (Group-II). The lungs of β-caryophyllene supplemented pneumonia mice were exhibited the reduced inflammation and tissue damages as seen in the control (Group-III). The standard drug azithromycin-treated pneumonia mice also showed reduced histological alterations (Group-IV).
Significance was determined by using one-way ANOVA followed by DMRT analysis. Values presented as a mean ± SD of triplicates (n = 6). IL-8, IL-1

Effect of \( \beta \)-caryophyllene on the expression of NF-\( \kappa \)B in the lung tissues of pneumonia induced mice. Values presented as a mean ± SD of triplicates (n = 6). Significance was determined by using one-way ANOVA followed by DMRT analysis. Note: * indicates p < 0.05 compared with control and ** indicates p < 0.05 compared with pneumonia induced group.

Neutrophils are the imperative immune cells that play crucial functions in innate immunity that contributes to the advancement of inflammatory reactions (Guo et al., 2015). The penetration of neutrophils is generally renewed as a major event in the \( M.pneumoniae \) activated pneumonia. Previous reports were mentioned that the status of neutrophils was severely augmented in both blood and BALF of corticosteroid-resistant refractory pneumonia (Zhang et al., 2016, Yan et al., 2016). The over-accumulation of neutrophils may lead to the hyperinflammatory condition due to the deliverance of pro-inflammatory regulators (Chen et al., 2016). The findings from the histological analysis of lung tissues of pneumonia stimulated mice exhibited severe neutrophils pene-

5. Conclusion

The original findings from this exploration confirmed the curative potential of \( \beta \)-caryophyllene against the \( M.pneumoniae \) trig-

The authors declare that they have no known competing finan-

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