Original Article

Phytochemical-induced reduction of pulmonary inflammation during *Klebsiella pneumoniae* lung infection in mice

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Abstract

Introduction: Curcumin, a polyphenol derived from the herb *Curcuma longa*, has number of antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities. Its anti-inflammatory property was here studied alone and in combination with clarithromycin in a mouse model of acute inflammation.

Methodology: A total of 80 mice divided into four groups were used. Mice receiving curcumin and/or clarithromycin were fed orally with curcumin (150 mg/kg/day) for 15 days prior to infection, whereas clarithromycin was administered orally (30 mg/kg/day) 12 hours post infection. Simultaneously, the control group receiving only infection but no treatment was also set up. Bacterial load estimation, histopathological examination and analysis of inflammatory parameters was performed on various days for all groups.

Results: Intranasal inoculation of bacteria resulted in significant increase in neutrophil infiltration along with increased production of various inflammatory mediators (malondialdehyde, myeloperoxidase, nitric oxide, TNFα) in lung tissue. Clarithromycin treatment significantly decreased the bacterial load and other inflammatory components in infected mice, but animals receiving curcumin alone or in combination with clarithromycin showed a much more significant ($p < 0.05$) reduction in neutrophil influx along with reduced levels of various inflammatory parameters. Though treatment with curcumin did not reduce the bacterial load, in combination with clarithromycin, both bacterial proliferation and lung tissue damage were checked.

Conclusions: Though clarithromycin, because of its associated side effects, may not be the preferred treatment, it can be used in conjunction with curcumin. The latter as an adjunct therapy will help to down regulate the exaggerated state of immune response during acute lung infection.

Key words:a cute lung injury; immunomodulator; inflammation; phytochemical.

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Introduction

Acute lung injuries during infections caused by nosocomial or community-acquired Gram-negative bacteria such as *Klebsiella pneumoniae* result in high mortality rates in the absence of therapeutic interventions [1]. Acute lung injuries are mainly characterized by increased proliferation, recruitment and activation of pro-inflammatory cells, production of various free radicals, and proinflammatory mediators in lungs [2,3]. This inflammatory process in its controlled state helps in fighting against infection, but the same inflammatory process due to the overwhelming activity of the immune system can also contribute to the pathogenesis, leading to deleterious effects on the organ involved. Various antibiotics have been used in the past to treat infections caused by *K. pneumoniae*,but treatment of acute bacterial infections such as pneumonia with existing antibiotics does not provide protection against organ damage generated by an active immune response [4]. It has been observed that antibiotics with antibacterial and anti-inflammatory action are good options in containing both the infectious agent as well as the harmful action of the overstimulated immune response. The macrolide group of antibiotics have been known to affect several pathways of inflammatory processes such as migration of neutrophils, oxidative burst in macrophages, and the production of pro-inflammatory cytokine [5,6], but because of the various side effects of antibiotics and the problem of ever-increasing antibiotic resistance, there has been a shift towards alternative or complementary ways of medicine. One such approach being explored is the use of herbal compounds. Due to its anti-inflammatory properties, turmeric-derived curcumin has been used for decades to curb inflammation [7-10]. Extensive research has shown that curcumin mediates its anti-inflammatory effect by down regulating inflammatory transcription

factors (such as nuclear factor kappa B), enzymes (such as cyclooxygenase 2 and 5 lipoxygenase), and cytokines (such as tumor necrosis factor, interleukin 1 and interleukin 6) [11].

Since both curcumin and clarithromycin have been shown to posses anti-inflammatory properties, their effect alone as well as in combination was evaluated in this study in a mouse model of pneumonia to curb infection-associated inflammation *invivo* due to *Klebsiella pneumoniae.*

Methodology

Bacterial strain

Klebsiella pneumoniae B5055 (NCTC 5055) initially obtained from Dr. Mathia Trautmann, Department of Medical Microbiology and Hygiene, University of Ulm, Germany, and maintained in the laboratory was used in the study.

Animals

A total of 80 infection-free BALB/c mice of both sexes, between six and eight weeks of age, weighing 20-25 grams, were procured from the central animal house of Panjab University, Chandigarh, India. Animals were kept in clear polypropylene cages and fed a standard antibiotic-free diet (Hindustan Lever Products, Kolkata, India) and water *ad libitum*. Temperature ranged between 18°C and 22°C, and relative humidity was maintained between 55% and 65%. The study was conducted after obtaining approval from the Animal Ethics Committee of Panjab University.

Experimental protocol

Mice were randomly divided into four groups; each group contained 20 animals. Group 1 (n = 20) consisted of mice suffering from *K. pneumoniae* B5055-induced lung infection without any standard drug treatment and receiving only normal saline orally. Group 2 (n = 20) included mice suffering from *K. pneumoniae* B5055-induced lung infection and receiving clarithromycin orally (30 mg/kg/day) 12 hours after bacterial inoculation into the lungs. Group 3 (n = 20) included mice which were fed curcumin (150 mg/kg) for 15 days and thereafter infected intranasally with *K. pneumoniae* B5055. Group 4 (n = 20) included mice that were fed curcumin (150 mg/kg) for 15 days, infected with *K. pneumoniae* B5055, and given clarithromycin treatment (30 mg/kg/day) orally 12 hours after bacterial inoculation into the lungs.

Induction of acute lung infection

Acute lung infection in mice was induced with *K. pneumoniae* B5055 following the original method of Held *et al*. [12] modified by Yadav *et al*.[13]. In brief, mice were given 10^4 colony-forming units/mL (CFU/mL) of *K. pneumoniae* B5055 intranasally in a volume of 50 µL while being held in an upright position without any anaesthesia. The animals were kept in this position for at least two minutes so as to allow proper transfer of inoculum in to the respiratory passage. The infection procedure was optimized in a separate set of experiments earlier. After infection, animals were euthanized on different days post infection by cervical dislocation, and their lungs were removed aseptically and examined for bacterial load and various other inflammatory parameters.

Quantification of bacteria in lungs

Lungs were removed aseptically and then homogenized in 1 mL of normal saline (0.85% NaCl, pH 7.2). Serial dilutions of the homogenized lung tissue were made, plated on nutrient agar (Himedia Labs, Mumbai, India) plates, incubated at 37°C for 24 hours, and quantitative bacterial counts were determined.

The lung homogenate from each mouse was also processed for malondialdehyde, myeloperoxidase, nitric oxide, and TNFα levels.

Malondialdehyde (MDA) estimation

The extent of tissue damage in terms of lipid peroxidation was estimated by measuring the amount of MDA using Ohkawa *et al*.'s method [14]. In brief, 0.2 mL of the lung homogenate was mixed with 4 mL of 1/11 N sulphuric acid, 1.5 mL of freshly prepared 0.8% thiobarbituric acid (TBA), and 0.2 mL of 8.1% sodium dodecyl sulphate. This mixture was then kept in a boiling water bath for 1 hour. After cooling the mixture under tap water, 5.0 mL of butanol pyridine (15:1) was added and the mixture was shaken vigorously. The contents were centrifuged at 4,000 rpm for 10 minutes; the upper organic layer was taken in a separate tube and its absorbance was taken at 532 nm. MDA concentration was expressed as µmole/mg of protein.

Myeloperoxidase (MPO) estimation

Pulmonary neutrophils infiltration was quantitated by measuring the MPO activity using a spectrophotometric method as described by Greenberger *et al*. [15]. Briefly, the lungs were removed, weighed to determine the wet weight, and

then homogenized in 2 mL of homogenizing solution containing 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyl trimethyl ammonium bromide and 5 mM EDTA. The homogenate was sonicated and centrifuged at 15,000 g for 15 minutes. The supernatant was mixed in a ratio of 1:15 with an assay buffer composed of 100 mM potassium phosphate buffer (pH 6.0), 0.167 mg/mL O-dianisidine hydrochloride, and 0.0005% hydrogen peroxide. MPO activity was assayed by measuring the change in absorbance at 460 nm from 0 to 4 minutes over intervals of 30 seconds each.

Nitric oxide (NO) estimation

Nitrite level was estimated in the lung homogenate according to the method of Tsai *et al*.[16]. Lung homogenate (0.1 mL) was mixed with 0.4 mL of phosphate buffer saline (0.1M, pH 7.2) and 2 mL of Griess reagent. Then 2 mL of trichloroacetic acid (TCA) was added, and the mixture was vortexed and incubated for 20 minutes. The mixture was then centrifuged at 14,000 g for 10 minutes and the absorbance of the supernatant was taken at 540 nm. Nitrite concentration was determined from the standard curve prepared with 0.1 mL of 100 μM sodium nitrite.

Estimation of TNFα levels

Assay for TNFα in the lung homogenates was performed by enzyme-linked immunosorbent assay (ELISA) using a commercially available cytokine kit, BD OptEIA Mouse TNFα ELISA Kit (BD Biosciences, Franklin Lakes, USA). In brief, lungs were homogenized in 1 mL of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.4). Homogenates were then incubated on ice for 30 minutes and centrifuged at 2,500 rpm for 10 minutes. Supernatants were then collected in sterile eppendorfs, passed through 0.45 μm pore size filter and stored at -60°C for the assessment of TNFα levels. The BD OptEIA test is a solid phase sandwich ELISA. The wells were read at 450 nm, and the concentration of TNFα in the test sample was determined from the standard curve prepared from the standard given along with the kit. The results were expressed as pg/mL of the TNFα released.

Histopathological examination

Lungs were removed aseptically, immersed in 10% formalin fixative, and processed for histological examination. The lung tissue was embedded in paraffin wax and cut into 4-6 μm thick sections using a microtome. After this, the sections were stained with hematoxylin and eosin for assessment of the degree of neutrophil infiltration.

Statistical analysis

The data are expressed as mean ± standard error of the mean (SEM). Results were analysed statistically by applying Student's *t*-test for comparing various parameters in treated and untreated control mice. Differences were considered statistically significant if P values were less than 0.05. Data was analyzed using Microsoft Excel 2007 software.

Results

Bacterial count in mice infected with K. pneumoniae B5055 and treated with curcumin and/or clarithromycin

No reduction in bacterial counts was observed in the mice administered only curcumin and infected with *K. pneumoniae* B5055 (Figure 1).The counts observed were similar to those observed in the control group. Bacterial counts in the lungs of mice treated with clarithromycin alone or in combination with curcumin showed a significant decrease ($p < 0.05$). By the seventh post-infection day, lungs became almost sterile after treatment, and no significant difference was observed in the counts of the former (0.351±0.212) as well as the latter group (0.591±0.203). This indicated no antagonistic action of curcumin over clarithromycin when used in combination.

Malondialdehyde (MDA) levels in mice infected with K. pneumoniae B5055 and treated with curcumin and/or clarithromycin

Raised levels of MDA after the first day of infection were observed in the lung homogenates of control mice infected with *K. pneumoniae* B5055 (Figure 2). Contrary to this, the MDA levels were significantly reduced ($p < 0.05$) the first day following infection in the animal groups treated with clarithromycin and curcumin alone or in combination. A similar trend was observed on all post-infection days in the clarithromycin- and curcumin-treated groups compared to what was observed in the control group. This decrease in MDA levels was found to be significant.

Figure 1. Bacterial count (Log_{10}CFU/mL) in the lungs of mice infected with *K. pneumoniae* B5055 and treated with curcumin and clarithromycin alone or incombination

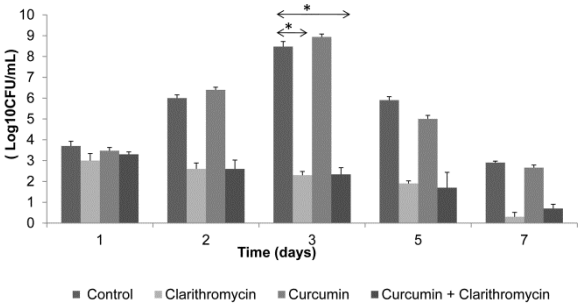


Figure 2. Malondialdehyde (MDA) levels (µmole/mg of protein) in the lung homogenates of mice infected with *K. pneumoniae* B5055 and treated with curcumin and clarithromycin alone or in combination

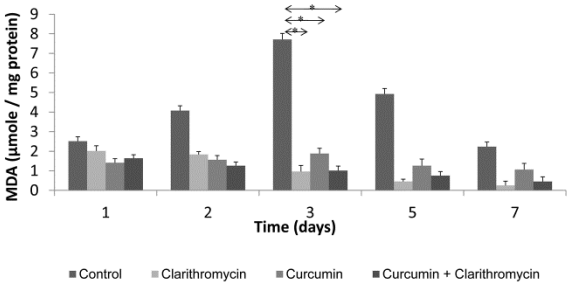


Figure 3. Nitric oxide (NO) levels (µmole) in the lungs of mice infected with *K. pneumoniae* B5055 and treated with curcumin and clarithromycin alone or incombination

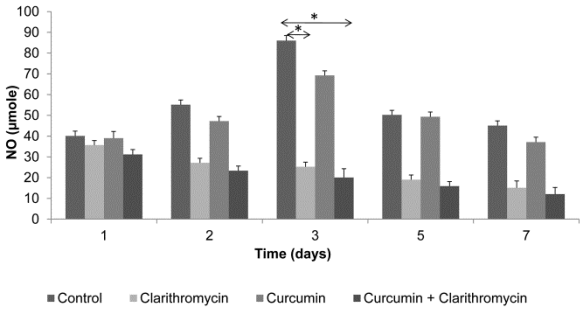


Figure 4. Myeloperoxidase (MPO) levels (MPO units) in lung homogenates of mice infected with *K. pneumoniae* B5055 and treated with curcumin and clarithromycin alone or in combination

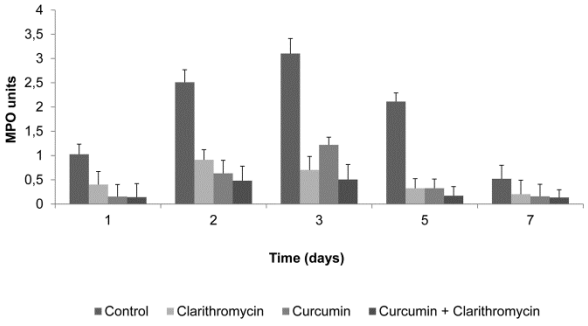
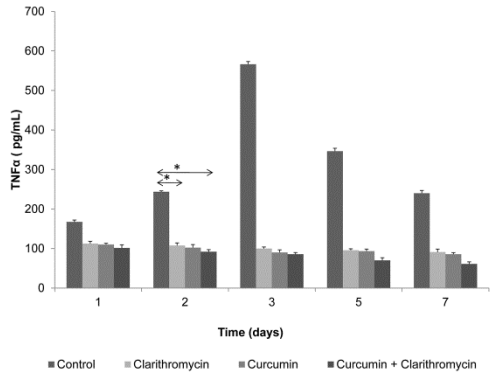


Figure 5. TNFα level (pg/mL) in the lungs of mice infected via intranasal route with *K.pneumoniae* B5055 and treated with curcumin and clarithromycin alone or in combination



Nitric oxide (NO) levels in mice infected with K. pneumoniae *B5055 and treated with curcumin and/or clarithromycin*

Decrease in nitric oxide levels was observed in the lung homogenates of clarithromycin-treated animals (Figure 3). This decrease was significantly lower than that obtained in the lung homogenates of animals treated with curcumin alone. The animals who received both curcumin and clarithromycin, however, showed a fourfold decrease in NO levels compared to the untreated control group, which was highly significant ($p < 0.05$) until the seventh post-infection day. In the control group, an increase in levels of NO was observed with time, and the maximum amount of NO was detected on third post-infection day**.** These levels declined with time but still remained higher until the seventh post-infection day.

Myeloperoxidase (MPO) levels in mice infected with K. pneumoniae *B5055 and treated with curcumin and/or clarithromycin*

Treatment of animal groups with curcumin and clarithromycin alone as well as in combination resulted in a decrease in MPO levels compared to the control group (Figure 4). The decrease observed was more significant ($p < 0.05$) in the group treated with curcumin and clarithromycin in combination than in the group of animals treated with either agent alone.

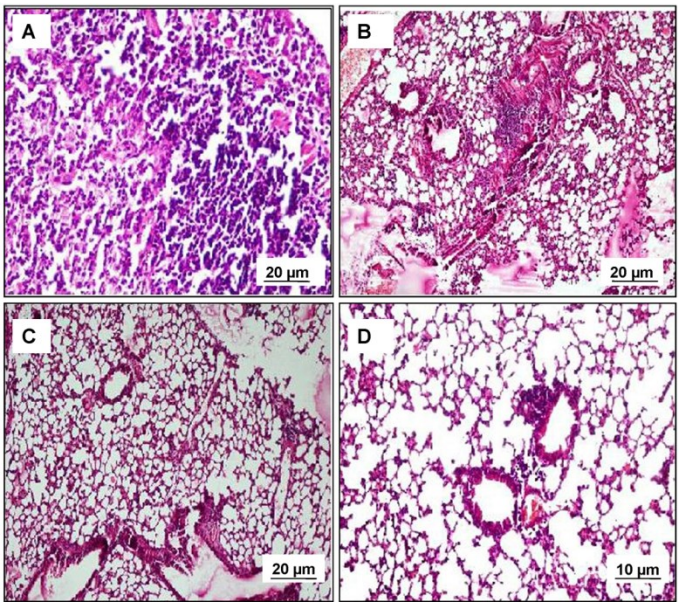
TNFα levels in mice infected via intranasal route with K. pneumoniae *B5055 and treated with curcumin and/or clarithromycin*

Treatment with clarithromycin alone or in combination with curcumin significantly ($p < 0.05$) reduced the TNFα levels in the lungs of mice infected with *K. pneumonia* B5055 compared to the levels observed in the lungs of control mice on the third post-infection day (Figure 5). The reduction in TNFα levels was seen on all post-infection days. This difference in the level of TNFα between the treated and the non-treated groups was significant ($p < 0.05$) beginning the second post-infection day onwards.

Histopathological changes in mice infected with K. pneumoniae *B5055 and treated with curcumin and/or clarithromycin*

On histopathological examination, lung alveoli of mice belonging to the control group were found to be filled with neutrophils (Figure 6a), compared to those suffering from pneumonia who were treated with curcumin or clarithromycin alone (Figures 6b and 6c). Although they showed significant reduction in neutrophil infiltration, signs of characteristic peribronchial inflammation were seen in the former group. On the other hand, clear alveoli were seen in the animals treated with clarithromycin in combination with curcumin (Figure 6d).

Figure 6. A) Lungs of mice suffering from *K. pneumoniae* B5055-induced pneumonia and treated with normal saline alone (control group) (40×); **B).** Lungs of mice given oral feeding for 15 days with curcumin followed by infection with *K. pneumonia*e (40×); **C)** Lungs of mice suffering from *K. pneumoniae* B5055-induced pneumonia and treated with clarithromycin alone (40×); **D)** Lungs of mice treated with both curcumin and clarithromycin (100×)



Discussion

A search for better immunomodulatory and anti-inflammatory agents is underway to find agents that are capable of modulating the immune system in such a way that any harmful action of the immune system is negated while the defensive property of the immune system remains intact. Working in this direction, we tried to control pulmonary inflammatorydamage in a mouse model of pneumonia using an herbal compound, curcumin, along with an antibiotic, clarithromycin, both of which have been shown to possess anti-inflammatory properties when tested individually. The intranasal inoculation of *K. pneumoniae* B5055 in BALB/c mice led to the establishment of infection as an increase in the bacterial load until the third day post-infection. The bacterial number started to decline thereafter, and lungs became sterile after the seventh post-infection day. Acute lung infection is characterized by a cascade of events resulting in activation of alveolar

macrophages and neutrophil infiltration. This gives rise to edema, tissue damage, respiratory dysfunction, release of various reactive oxygen species (ROS), and proinflammatory cytokines [17]. Thus, levels of various biomarkers of inflammation or tissue injury such as MPO, MDA, NO, and TNFα were studied. They reached maximum levels on the third post-infection day and subsequently declined, confirming induction of acute inflammation. Previously, Punithavatihi *et al*. [18] demonstrated that dietary supplementation with 200 mg/kg curcumin led to significant inhibition of neutrophil influx, superoxide generation, and MPO activity in rats with lung infections.Similar results were obtained in our study, as pre-treatment of animals with curcumin 15 days prior to inoculation of bacteria in the lungs via the intranasal route showed significant reduction in lung MPO, MDA, NO, and TNFα levels without any effect on the bacterial number. This suggests that, despite not being antibacterial in nature, curcumin has potent anti-inflammatory properties. Clarithromycin treatment, on the other hand, significantly reduced ($p < 0.05$) both bacterial load and various inflammatory parameters such as MDA, MPO, NO, TNFα, and neutrophil infiltration in the lungs of infected mice. Previously, Yoshida *et al*. [19] also observed that macrolide antibiotics promote monocyte differentiation to macrophages and that the chemically modified macrolide antibiotic EM-A leads to an increased macrophage spreading effect. A decline in the levels of various inflammatory markers such as NO, MDA, MPO, TNFα, and neutrophil infiltration following treatment withclarithromycin during acute lung injury, otitis media, and various other pathological conditions has also been observed by different workers [20-23].

In the present study, when curcumin-fed mice were treated with clarithromycin to control *K. pneumoniae* B5055-induced lung infection, no antagonistic interaction between curcumin and clarithromycin was observed (Figure 1). Their combination not only reduced the bacterial counts significantly ($p < 0.05$), but also reduced free radical generation, oxidative stress, lipid peroxidation, neutrophil infiltration, and lung injury occurring as a result of acute lung infection induced inflammation. This indicates that the combination of both is more effective than either agent alone. Curcumin has been approved as safe by the United States Food and Drug Administration, and the World Health Organization. Phase I and II clinical trials show that people can tolerate curcumin at doses as high as 8 g/day [24]. This can help in developing novel strategies incorporating curcumin for the prevention and treatment of acute lung infection along with antibiotics. The need for such immunomodulators as well as anti-inflammatory agents seems to be increasing in the field of nosocomial acute lung infections due to associated acute lung injuries. Curcumin binds to over a dozen different cellular proteins and enzymes, scavenges free radicals, and exerts antioxidant and anti-inflammatory activity [25]. This non-nutritive phytochemical is pharmacologically safe; it has been consumed as a dietary spice at doses up to 100 mg/day for centuries [26]. Thus, since it has no side effects, is cost effective, and is a part of our daily diet, it can prove to be a very good prophylactic as well as therapeutic adjunct along with antibiotics to curb inflammation [11].

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