Abstract

Pneumonia caused by Klebsiella pneumoniae is important due to its high morbidity and mortality. This infection causes acute inflammation in the lung is characterized by increased activity of neutrophils, generate oxy free radical and decreased the endogenous anti oxidant defense system.

CSE1034 is a novel fixed dose combination drug of ceftriaxone plus sulbactam with VRP1034. The aim of this investigation was to compare the efficacy study of CSE1034 drug vs ceftriaxone alone in pneumonia induced rat model. For pneumonia infection in animal model, doses were standardized at concentration 10° to 10° CFU/ml of Klebsiella pneumoniae.

Total thirty two male rats (150 ± 5 g) were randomly selected and divided into four groups of eight animals each. Group I was normal saline treated; group II was pneumonia infected; group III was infected plus ceftriaxone treated and group IV was infected plus CSE1034 treated. Pneumonia infection was induced in all group except group I via intranasal instillation, at concentration (log 10 CFU/ml) for 15 days. Infection was confirmed by raised body temperature, bacterial count, cell count and cytokine (TNF-α, IL-6) parameters in blood. After conformation of infection, CSE1034 and ceftriaxone drugs treatment were stared for 15 days. At the end experiment, blood and lung tissue were collected and measured the biochemical and enzymic parameters in all group.

The finding showed that a significant decrease lactate dehydrogenase activity, malonaldialdehyde, total protein, albumin, nitrate, tumor necrosis factor-α, interleukin-6 levels and bacterial count along with increase reduced glutathione level in lung homogenate of CSE1034 treated group as compared to pneumonia induced and ceftriaxone treated groups. These findings suggested that CSE1034 is effective than ceftriaxone which reduced bacterial count and enhanced endogenous antioxidant status along with reduces, inflammatory response during pneumonia infection.

Keywords: CSE1034; Cytokines levels; Endogenous antioxidant enzymes; Klebsiella pneumoniae; Malonaldialdehyde; Pneumonia

Introduction

Klebsiella pneumoniae is an important cause of both community-acquired as well as nosocomial lung infection. Pneumonia caused by K. pneumoniae organism has a rapidly progressive clinical course which is often complicated by multilobular involvement and lung abscesses [1,2]. Several epidemiological studies have showed that the frequency of nosocomial infections caused by Klebsiella species increased substantially over the last 20 years [3,4]. Nosocomial pneumonia (NP) is currently the second most common and leading cause of death [5]. Bacterial infection causes the acute inflammation in the lung is characterized by increased activity of neutrophils and generate oxy free radical [6].

Ceftriaxone is a third generation cephalosporin class of beta-lactam drug with potent bactericidal activity against a wide range of gram positive and gram negative bacteria [7]. The antibacterial activity of ceftriaxone is due to inhibition of cell wall synthesis [8]. CSE1034 is a novel fixed dose combination of ceftriaxone plus sulbactam with VRP1034. Sulbactam is potent and highly specific inhibitors of a wide range variety of beta lactamase produced by common gram positive and gram negative aerobes and anaerobes [9]. It is a molecule which inhibits beta lactamase, an enzymes produced by bacteria that destroys the antibiotics. VRP1034 (under patent) used as a third vector for their syngenic effect and play a significant role for reduction of toxicity. It is having a antimicrobial, chelating and antioxidant properties. Combination therapy is widely used empirically in life-threatening infections, when more than one antimicrobial is preferred if a single one is not expected to have a spectrum broad enough to cover all potential pathogens. Hence the aim of this study was to determined the comparative efficacy CSE1034 vs ceftriaxone alone drugs in Klebsiella pneumoniae induced pneumonia induced rat model.

Materials and Methods

Chemicals and drugs

All the biochemicals used in the present study were procured from Sigma, St. Louis, MO, USA. Other chemicals, purchased locally, were of analytical grade. Biochemical kits such as LDH, albumin tec were procured from Reckon diagnostic private limited, Baroda India.

Ceftriaxone (1000 mg) and CSE1034 (1500 mg) drugs were obtained from Venus Remedies Ltd. Panchkula, India as gift samples. CSE1034 is a novel research drug of Venus Remedies Ltd and it is under patent. The ratio of CSE1034 drug was 2:1 respectively.

Bacterial strain

Klebsiella pneumoniae organism (MTCC 109) were procured from Institute of microbial technology (CSIR laboratory) sector 39-A, Chandigarh India.
Antimicrobial susceptibility test (AST)

AST of CSE1034 and ceftriaxon drug were performed according to CLSI guidelines [10].

Bacterial inoculum

Bacterial strain maintained on nutrient agar slant, were grown in static culture in nutrient broth at 37°C for 18 to 20 hours. Organism were harvested and washed to 3-4 times by centrifuged at 2500 xg for 20 minutes and suspended in 0.2 M phosphate buffer saline (pH 7.2) for the 1 x 10⁵ CFU (colony form unit) per ml.

Bacterial dose response for induction of pneumonia

The optimal dose required for establishing pneumonia in animals was standardized prior to studying the course of pneumonia. For the bacterial dose response in animals, total 30 male rats were selected and divided into five groups of six rat each. A single dose range from 10⁶ to 10⁷ CFU/ml of K. pneumoniae bacteria culture were given to group II to IV via intranasal route and blood samples were collected from each animals via retro-renal vein in sterile tubes at every 6 hours for 24 hours. Measured the bacterial count in the blood sample rest part of blood samples were centrifuged at 6000 rpm and plasma samples were collected and store at -80°C for measurement of tumor necrosis factor-a and interleukin-β.

Animals

The experiment was carried out after approval Institutional animal ethics committee (IAEC/CS/10/2011). Total 32 male rats (150 ± 5 gm) were selected for this experiments. The rats were fed standard pelleted diet and water ad libitum. The test room was air conditioned with temperature 23 ± 2°C, humidity 65 ± 5%, and with artificial fluorescent light (12 hours of light and dark, respectively).

Induction of pneumonia

For intranasal instillation of the bacterial inoculum, the method of Held et al was employed [11]. 50 μl of log 10⁶ CFU/ml bacterial inoculum was instilled into the nasal opening while holding the animals upright for 15 days. The bacterial inoculum was given to all animals twice daily (at 9.00 a.m. and 5.00 p.m.) Total 24 animals (Eight rats in each group) were infected by confirmation of increased body temperature cell count (WBC) and presence of bacterial count in blood sample. Group I (eight rat) was non infected and treated with 0.9% NaCl.

Treatment

Total 32 rats were divided into four groups. Each group have eight animals as given below:-

- Group I (n=8) Control normal saline treated group
- Group II (n=8) K. pneumoniae infected (log 10⁶ CFU/ml) group
- Group III (n=8) Infected + Ceftriaxon treated group (103.33 mg/Kg/day body weight)
- Group IV (n=8) Infected + CSE1034 treated group (155.0 mg/Kg/day body weight)

After confirmation of pneumonia infection, CSE1034 and ceftriaxon drugs were administered via intravenous route for 15 days treatment and blood samples were collected every 3rd day interval for 15 day and measured the bacterial count. At the end of experiment, animals were sacrificed, lung tissues were collected from each group and immediate photograph of lung tissue were taken for observation of gross changes in each group. Lung tissue was sectioned into two halves. One half of each lung was placed in a sterile tube and prepared the homogenate and other half part of lung tissue was used for histological examination.

Homogenate preparation

10% lung homogenate was prepared in sterile phosphate buffer-NaCl solution containing 0.15 mol/L NaCl in 0.05 mol/L NaH₂PO₄-NaH₂PO₄ buffer (pH 7.2) for the measurement of bacterial count and rest part of homogenate was left for at least 1 hr at 0°C before the estimation of enzyme assay other biochemical parameters.

Reduced glutathione (GSH) assay

Reduced glutathione was estimated by the method of Ellman [12]. 0.5 ml tissue homogenate was mixed with equal amount of 5% (w/v) TCA reagent and kept for 10 min at room temperature, proteins were precipitated and filterate was removed carefully after centrifuge at 3500 rpm for 15 minutes. Take 0.25 ml filtrate was taken and added to 2.0 ml of Na₂HPO₄ (4.25%) and 0.04 ml of DTNB (0.04%). A blank sample was prepared in similar manner using double distilled water in place of the filtrate. The pale yellow color was developed and optical density was measured at 412 nm by spectrophotometer.

Measurement of lipid peroxidation

Lipid peroxidation was measured according to Ohkawa et al. [13]. It was determined by thio barbituric reaction. The reaction mixture consisted of 200 μl of lung tissue homogenate, 0.20 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of acetic acid (20%, pH 3.5), 1.5 ml of 0.8% thio barbituric acid (TBA) and water to make up the volume to 4.0 ml. The tubes were boiled in water bath at 95°C for one hour and cooled immediately under running tap water. Added 1.0 ml of water and 5.0 ml of mixture of n-butanol and pyridine (15:1 v/v) and vortexed. The tubes were centrifuged at 3500 rpm for 30 minutes. The upper layer was aspirated out and optical density was measured at 532 nm Molar extension coefficient 1.56 x 10⁵ Cm⁻¹ was used for calculation.

Determination of myeloperoxidase

Myeloperoxidase level was determined by O-dianisidine method with slight modification Kurutas et al. [14]. The assay mixture consisted of 0.3 ml of sodium phosphate buffer (0.1 M; pH 6.0) 0.3 ml of H₂O₂ (0.01 M), 0.2 ml of O-dianisidine (0.02 M) (freshly prepared) in distilled water and made to a final volume of 3.0 ml with water. The reaction was started by the addition of 0.050 ml of tissue homogenate. The change in absorbance was recorded at 460 nm wavelength. One unit of peroxidase activity equaled the amount of enzyme decomposing 1 μmol of hydrogen peroxide per minute at 25°C. Decomposition of hydrogen peroxide was calculated from the oxidation of o-dianisidine using an absorption coefficient of 11.3/mM/cm at 460 nm.

Nitric Oxide determination

The nitrite level was estimated in the lung homogenate according to method of Tasi et al. [15]. The lung homogenate (150 μl) was mixed with 0.4 ml phosphate buffer saline (0.1M, pH 7.2) and added 2.0 ml Griess reagent. Then 2.0 ml of 5% TCA solution was added and mixed properly by vortex shaker and kept for incubation for 15-20 minutes. After incubation, the reaction mixture was centrifuged at 14000 xg for 20 minutes and supernatant was taken carefully in other clean tube and absorbance was measured at 540 nm. The concentration of nitrite
was determined from stranded curve prepared with 0.1ml of 100 μM sodium nitrite.

Measurement of Biochemical parameters

Biochemical parameters such lactate dehydrogenase enzyme activity (LDH) and albumin levels were assayed by kit on fully automatic biochemical analyzer. Total protein in the lung homogenate was assayed by Lowery method [16].

Cytokines assay

The Concentration of tumor necrosis factor-α (TNF-α), interleukin-β (IL-β) and interleukin-6 (IL-6) were measured with commercial enzyme-linked immunosorbent assay (ELISA) kits purchased from Invitrogen SanJose CA, USA. The assays were performed according to protocol recommended by the manufacturer's.

Histopathological analysis

Tissue samples from lung, stored at 10% formalin buffer were trimmed, dehydrated, embedded in paraffin, cut into 5 μm sections and stained with hematoxylin and eosin. Histological changes were observed with a light microscope.

Statistical analysis

Data are expressed as means ± SD. Data comparisons were carried out using one way analysis of variance followed by Tukey’s post test to compare the means of control group vs pneumonia induced groups and pneumonia induced group vs CSE1034 and ceftriaxone treated group. P < 0.05 was considered as statistically significant.

Results

Bacterial count at different time intervals and doses response on cytokine parameters

In the present investigation, there was significantly (p < 0.001) enhanced bacterial count in the blood of 10⁶ CFU/ml dose concentration in comparison to other doses at every six hours. Bacterial count was found almost equal in 10⁴, 10⁵ CFU/ml at 12 hours whereas at 18 hours, the bacterial count was slightly reduced at dose 10⁵ CFU/ml but at 24 hours, the count was increased at dose 10⁶ CFU/ml. At dose 10⁷ CFU/ml, there were no count observed at different time intervals. Similarly TNF-α and IL-6 levels were also found significantly (p < 0.001) elevated in the blood of 10⁶ CFU/ml dose concentration in comparison to control group and 10⁴, 10⁵, and 10⁶ CFU/ml bacterial doses. TNF-α level was slightly increased in dose 10⁶ CFU/ml in comparison to 10⁴, 10⁵, 10⁶ CFU/ml bacterial doses. IL-6 level was slightly significantly increased only in 10⁴, 10⁵ CFU/ml bacterial doses as compared to control and 10⁶ CFU/ml (Figure 1-3).

Body temperature and white blood cells

Figure 4 and 5 showed a significantly (p < 0.001) raised body temperature and white blood cell count (WBC) in the pneumonia induced group as compared to control group after 15 days intranasal exposure of *Klebsiella pneumoniae* microorganism. The body temperature and WBC count were significantly reduced (p < 0.05); (p < 0.01) in ceftriaxone treated group as well as (p < 0.001) in CSE1034 treated group after 15 days treatment in comparison to pneumonia induced group. When both treated groups were compared between each other, the body temperature and WBC count were significantly (p < 0.05); (p < 0.01) lowered in CSE1034 treated group.

Gross changes in lung tissue

The infected lung tissue shows severe congestion, consolidation of the cardiac and apical lobes, and tracheal congestion as compared to normal. Gross mottling of paranchyma was also seen in infected group. In ceftriaxone treated group, lobes were flabby with petechial hemorrhages on the borders whereas in CSE1034 (ceftriaxone plus sulbactam with VRP1034) treated group, the lobes were apparently normal in appearance after 15 days of treatment (Figure 6).
Anti-microbial effect

In vitro anti-microbial effect of drugs showed that there was significantly (p < 0.001) increased about 53.6% zone of inhibition in CSE1034 treated drug against Klebsiella pneumoniae. The zone diameter of CSE1034 treated plate was found higher in comparison to ceftriaxone alone treated plate (Figure 7).

Bacterial count in blood and lung tissue

There was progressively increased bacterial count in the blood of pneumonia induced group at 3rd day interval up to fifteen days as compared to control group. After treatment with ceftriaxone and CSE1034 drugs for fifteen days, the bacterial count was gradually reduced in both treated groups. When both treated groups were compared to each other, the reduction of bacterial count was found better in CSE1034 treated group in comparison to ceftriaxone treated group after fifteen days. The bacterial count was found (p < 0.001) significantly higher in the lung tissue of pneumonia induced group after fifteen days exposure of K. pneumoniae microorganism. After treatment with respective drugs for fifteen days, the bacterial count was significantly (p < 0.01); (p < 0.001) reduced in the lung tissue of both treated group. But when both treated group was compared to each other, the reduction of bacterial count was found (p < 0.001) better in the CSE1034 treated group (Figure 8 and 9).

Effect on Biochemical parameters

There were significantly elevated (p < 0.001) protein and albumin levels in pneumonia induced group as compared to control group after fifteen days exposure of K. pneumoniae microorganism. After treatment with ceftriaxone and CSE1034 drugs for fifteen days, the protein level was significantly (p < 0.001) reduced in both treated groups whereas in case of albumin level, there was less significantly (p < 0.01) reduced in ceftriaxone treated group and highly significantly (p < 0.001) reduced in CSE1034 treated group. When ceftriaxone treated group was compared with CSE1034 treated group, both levels were significantly (p < 0.001) reduced in the CSE1034 treated group after fifteen days treatment (Table 1).

Lactate dehydrogenase and myeloperoxidase activities were significantly (p < 0.001) increased in the lung homogenate of pneumonia induced group as compared to control group. After treatment with respective drugs for fifteen days, these enzyme activities were reduced significantly (p < 0.001) in both treated groups as compared to control group.}

Figure 4: All result were mean ± SD of eight animals. Tukey’s post test was analyzed statistical significant between control group vs infected group and infected group vs drug treated group.

Figure 5: Status of total white blood count levels in K. pneumoniae induced drug treated group after 15 days treatment. All results were mean ± SD of eight animals. Tukey’s post test was analyzed statistical significant between control group vs infected group and infected group vs drug treated group. Where *** is highly significant (p<0.001), ** is significant (p<0.01), Ns; not significant (p>0.05).

Figure 6: Morphological changes in the lung tissue after intranasal instillation of K. pneumoniae and drug treated group. A image showing normal colour and gross morphology of lung; B image showing severe diffuse congestion and atelectasis of lung lobes due to infection caused by Klebsiella pneumoniae; C image showing moderate atelectasis of lung lobes after treatment with ceftriaxone treatment; D image showing normal gross morphology and decrease in congestion and atelectasis of lung lobes after treatment with CSE 1034.

Figure 7: In vitro Antimicrobial effect of Ceftriaxone and CSE1034 in K. Pneumoniae (MTCC-109). All data are Mean ± SD of three culture plate of ceftriaxone and CSE 1034 treated. The zone of inhibition of 53.6% increased in CSE1034 treated plates as compared to ceftriaxone treated plates.
were significantly \( (p < 0.01); (p < 0.001) \) reduced in the both treated group. When ceftriaxone treated group was compared with CSE1034 treated group, the LDH activity was insignificant \( (p > 0.05) \) decreased along with significant decreased \( (p < 0.001) \) myeloperoxidase activity in the lung tissue of CSE1034 treated group (Table 1 and Figure 10).

Reduced glutathione level was significantly \( (p < 0.001) \) lowered in the pneumonia induced as compared with control group after fifteen days exposure of \( K. \) pneumoniae. After intravenous treatment with ceftriaxone and CSE1034 drugs for fifteen days, the GSH level was found significantly \( (p < 0.05) \) increased in ceftriaxone treated group as well as significantly \( (p < 0.001) \) elevated in CSE1034 treated group. When both treated group was compared between each other, the level was found to be significantly \( (p < 0.001) \) increased in the CSE1034 treated group. Nitric oxide and malonalldialdehyde levels were significantly \( (p < 0.001) \) higher in the lung tissue of pneumonia induced group when compared with control group. These levels were reduced \( (p < 0.001) \) significantly in both treated groups after treatment with respective drugs for fifteen days. These levels were significantly reduced \( (p < 0.001) \) in the CSE1034 treated group when compared with ceftriaxone alone treated group (Table 1).

The inflammatory parameters such as TNF-α and IL-6 were significantly \( (p < 0.001) \) higher in the lung tissue of pneumonia induced group. After treatment with ceftriaxone and CSE1034 drugs for fifteen days, these parameters were significantly \( (p < 0.001) \) lowered in the both treated groups. On comparison among both treated groups, these levels were significantly \( (p < 0.001) \) found lower in the CSE1034 treated group after fifteen day treatment (Table 1).

The microscopic examination of lungs showed normal histological picture in control group whereas in infected group, lung sections showed edema formation along with hemorrhages, congestion and severe lymphocyte and macrophage infiltration were observed. In ceftriaxone treated group, moderate edema formation, congestion and lymphocytic infiltration observed in the lung tissue. The CSE1034 treated group showed mild congestion were seen in the lung tissue (Figure 11).

Discussion

Klebsiella pneumoniae is a gram-negative bacteria and is a member of Enterobacteriaceae family. It is an opportunistic pathogen that causes community-acquired and nosocomial infections. Infections caused by it ranges from mild urinary infections to severe pneumonia with a high rate of mortality and morbidity [17,18]. The intranasal route of this microorganism causes acute inflammation in the lung.
of beta lactam antibiotic while CSE 1034 is a combination of third generation β-lactam antibiotic with sulbactam beta lactamase inhibitor. VRP1034 is a third vector which showed chelating property that competes with microorganism for any of the trace iron and Ca ions which are essential to the maintenance of their life cycle. It penetrates the cell membrane and open the Ca channel and enhanced the concentration of combination drug in bacterial cell leading to cell death. The role of VRP1034 is to bind with essential divalent metal ions and hence make them unavailable to the bacteria for cellular replication and growth. The sensitivity of bacteria to VRP1034 used to enhance the susceptibility of bacteria to antibiotics by destabilizing the cell wall structure. So in our study, CSE1034 drug showed better antimicrobial effect then ceftriaxone alone (increased zone of inhibition) against gram-negative bacteria. It means combination of drug CSE1034 showed synergistic effect and better anti-microbial effect than ceftriaxone alone. The levels of malondialdehyde, cytokines, myeloperoxidase and lactate dehydrogenase activity were also significantly decreased along with increased the reduced glutathione level in the combination drug of CSE1034 treated group than ceftriaxone alone after fifteen days treatment. The results also indicated that CSE1034 showed free radical scavenger property than ceftriaxone alone which reduced free radical mediated tissue injury. Various studies have reported that cephalosporin and sulbactam antibiotics showed free radical scavenger property and their effect on inhibition of neutrophil function [25]. The conclusion of this study revealed that a novel CSE1034 showed better antimicrobial effect and free radical scavenger property which inhibits the free radical tissue injury and inflammatory response in the lung during pneumonia infection and is a safer and more effective drug.

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