Original Article

Cefazolin Plus Minocycline against a Clinical Isolate of *Vibrio vulnificus*: In Vitro and Animal Studies

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SUMMARY: Cefotaxime plus minocycline has been shown to have synergistic activity against *Vibrio vulnificus*; however, the clinical role of cefazolin in combination with minocycline in immunocompromised hosts has not been established. Therefore, antimicrobial susceptibility of the *V. vulnificus* clinical isolate Vv05191 was studied by the agar dilution method. Antibacterial activity of cefazolin, minocycline, and a combination of the two drugs was investigated by time-kill studies in vitro and further examined for therapeutic efficacy in a murine model. When cefazolin at a combination of 4 mg/L (1/2 × MIC) was combined with minocycline at a concentration of 0.03 mg/L (1/2 × MIC), sustained inhibitory activity was noted until 24 h. In BALB/cByJ mice with cyclophosphamide-induced neutropenia, an inoculum of 1.5 × 10⁸ CFU caused death within 96 h when the infected mice were treated by cefazolin (400 mg/kg every 3 h), while 6.3% of mice survived when treated by minocycline (4 mg/kg stat, then 2 mg/kg every 12 h). However, 62.5% of mice survived for 96 h when mice were treated by cefazolin (400 mg/kg every 3 h) plus minocycline (4 mg/kg stat, then 2 mg/kg every 12 h) (P = 0.002, log rank test). In conclusion, cefazolin in combination with minocycline exhibits in vitro synergistic antibacterial activity against *V. vulnificus* and provides a therapeutic advantage in neutropenic mice with *V. vulnificus* infection.

INTRODUCTION

*Vibrio vulnificus* is primarily associated with a severe, distinctive soft tissue infection or septicemia, or both, especially in immunocompromised patients with conditions such as liver cirrhosis, adrenal insufficiency, malignancy, or diabetes (1–4). Most *V. vulnificus* isolates are in vitro susceptible to a variety of antibiotics, including newer fluoroquinolones (5–10). Two previous in vitro and in vivo studies by Chuang et al. have demonstrated that combination therapy of cefotaxime and minocycline was more advantageous than either antibiotic alone for the treatment of severe experimental murine *V. vulnificus* infections (11,12).

In a previous animal study of *V. vulnificus* infection, immunocompetent mice were experimentally treated by a third-generation cephalosporin combined with minocycline (12). However, most lethal *V. vulnificus* infections occur in immunocompromised patients. When reviewing a published clinical study (4), we found some successful experiences in treating *V. vulnificus* infections with cefazolin alone or in combination with other drugs. Therefore, in the present study, we examined the in vitro susceptibility of *V. vulnificus* to cefazolin and minocycline, to evaluate the efficacy of combination therapy in a neutropenic mouse model.

MATERIALS AND METHODS

Minimal inhibitory concentration (MIC): The MIC values of minocycline and cefazolin were measured by the agar dilution method as previously described by National Committee for Clinical Laboratory Standards (13). Standard powders of both drugs were purchased from Sigma-Aldrich (St. Louis, Mo., USA). The drugs were incorporated into agars in a series of concentrations in two-fold dilution, ranging from 0.00375 to 256 mg/L. Minocycline was dissolved in 0.1 N NaOH solution, and cefazolin was dissolved in sterile water. *Escherichia coli* ATCC 25922 was used in each run as a control.

Bacterial strains: A clinical isolate of *V. vulnificus*, Vv-05191, was randomly selected from a cirrhotic case with lethal *V. vulnificus* bacteremia and necrotizing fasciitis. The organism was stored at −70°C in Protect Bacterial Preservers (Technical Service Consultants Ltd., Heywood, Lancashire, England) before use.

Inhibitory activities of cefazolin, minocycline, and a combination of the two drugs in time-kill studies: Bacterial concentrations were diluted to around 5.0 × 10⁵ CFU/mL in 25 mL of fresh Mueller-Hinton broth. The drug concentrations of minocycline or cefazolin in the following time-kill studies were adjusted to the 1/2 or 1 × MIC of each drug as indicated. Each flask was incubated at 37°C. Bacterial counts were measured at selected time intervals of 0, 2, 4, 6, 8, 12, 24, 30, 36, and 48 h by enumerating the colonies in 10-fold serially diluted specimens of 100-μl aliquots plated on nutrient agar (Difco Laboratories, Detroit, Mich., USA). All experiments were carried out in duplicate.

**In vivo mouse study:** Female, pathogen-free, inbred BALB/cByJ mice (Animal Center, National Science Council, Taipei, Taiwan), weighing on average 20 g (6–10 weeks old), were used throughout this study. A bacterial suspension in a volume of 0.1 mL was delivered intraperitoneally into each mouse. Minocycline was administered intraperitoneally at doses of 4 mg/kg stat and then 2 mg/kg every 12 h, and cefazolin was administered at doses of 400 mg/kg every 3 h, as previously described (14–16). Cefazolin was administered at 10 times the human dose (40 mg/kg) every 3 h due to the high body surface area and metabolic rate of mice and our in vivo pharmacokinetics data (data not shown). Intraperitoneal...
injection of cyclophosphamide (Sigma) at daily doses of 100 mg/kg for 3 days was used to induce neutropenia in mice (17).

There were four experimental groups, including the control group (no antimicrobial agent was given), and the cefazolin, minocycline, and cefazolin-minocycline treatment groups. There were 9 or 16 mice in each group, and the experiments were performed twice, one in non-neutropenic mice and one in neutropenic mice. In performing the animal experiments we complied with all relevant national guidelines of the Republic of China and with the Chi-Mei Foundation Medical Center Animal Use Policy.

Statistical methods: Data analyses were performed with SPSS for Windows 10.0 (SPSS Inc., Chicago, Ill., USA). The log-rank test was applied to compare the effect between different groups. A P value of less than 0.05 was considered to be statistically significant.

RESULTS

Time-kill studies: The MIC of cefazolin and minocycline for Vv05191 was 8 and 0.06 mg/L, respectively. When Vv05191 at an inoculum of 5 × 10^6 CFU/mL was incubated with minocycline at the concentration of 1/2 × MIC (0.03 mg/L) or 1 × MIC (0.06 mg/L), the bacterial growth was inhibited temporarily, but resumed at 12 or 24 h, respectively (Figure 1). At the cefazolin concentration of 1 × MIC (8 mg/L), there was sustained bactericidal activity lasting for 48 h. With the combination of cefazolin and minocycline, both at the concentration of 1/2 × MIC, the inhibitory activity lasted for 24 h and was synergistically bactericidal, defined as a reduction of viable bacterial colonies by at least two orders of magnitude in comparison with either drug alone at 24 h.

Therapeutic efficacy of combination therapy in the murine model: Two rounds of animal experiments were performed, and the initial inoculum was 4.5 × 10^8 and 1.5 × 10^8 CFU, respectively. Neutropenia was induced at round 2. Neutrophil counts in three normal mice at 7–8 weeks old were 2,750/ul, 3,140/ul, and 2,980/ul, respectively, and in mice receiving cyclophosphamide were 35/ul, 55/ul, and 20/ul, respectively (Table 1). Without antimicrobial therapy or with cefazolin therapy, no mouse survived at 5 days after intra-peritoneal inoculation of Vv05191. Of non-neutropenic mice treated by minocycline (2 mg/kg every 12 h) in combination with cefazolin (400 mg/kg every 3 h), the survival rate was similar to that of mice treated by minocycline (77.8% versus 66.7%, P = 0.73). However, the survival rate of neutropenic mice treated by the combination regimen was significantly higher than that of neutropenic mice treated by minocycline only (62.5% versus 6.3%, P = 0.002) (Table 1).

DISCUSSION

The clinical course of septicemic patients with V. vulnificus is often rapid progression, and over 50% of such patients die within 48 h of hospitalization (4). The previous study showed that cefotaxime combined with minocycline was in vitro active against V. vulnificus and was effective in a mouse model (12). Clinical experiences also support the use of minocycline plus cefotaxime for severe V. vulnificus infection (4). Due to the broad spectrum and higher cost of cefotaxime, it was our intent to determine whether cefazolin has similar inhibitory activity against V. vulnificus. Moreover, since severe V. vulnificus infection often occurs in immunocompromised patients, it is important to investigate the therapeutic effect of cefazolin plus minocycline in neutropenic animals before its application in clinical practice.

The in vitro antibacterial effects of cefazolin and cefotaxime against V. vulnificus, as reflected by the time-kill method, were greatly different. With cefotaxime at the concentration of 1 × MIC (0.03 mg/L) in an earlier report, the number of bacterial colonies returned to the initial inoculum within 8 h (11), but in the present study with cefazolin at 8 mg/L (1 × MIC) there was sustained inhibitory activity for at least 48 h. Such a result is probably related to the different bacterial strains and mouse species tested. Despite of the obvious difference in in vitro antibacterial activity between cefotaxime and cefazolin, the therapeutic efficacy of both drugs for mice infected by a large inoculum of V. vulnificus was poor, i.e., all infected mice died at 96 h (12).

In time-kill studies, some differences in antibacterial effects between minocycline and cefazolin were noted. At the MIC, the inhibitory effect of minocycline persisted for about 24 h, but the inhibitory effect of cefazolin alone persisted for more than 48 h. At the 1/2 × MIC of both drugs, synergism was found and the inhibitory effect persisted for 24 h. In the mouse model, the survival rate of the combination group

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Table 1: Survival rates of BALB/c mice intraperitoneally infected with Vibrio vulnificus 05191 at 96 h after treatment with saline (control), cefazolin, minocycline, or a combination of the two drugs

<table>
<thead>
<tr>
<th>Initial inocula (CFU)</th>
<th>Cyclophosphamide-induced neutropenia</th>
<th>Surviving/Total mouse no. (%)</th>
<th>Control</th>
<th>Cefazolin</th>
<th>Minocycline</th>
<th>Cefazolin + minocycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5 × 10^8</td>
<td>No</td>
<td>0/9 (0)</td>
<td>0/9 (0)</td>
<td>6/9 (66.7)</td>
<td>7/9 (77.8)</td>
<td></td>
</tr>
<tr>
<td>1.5 × 10^8</td>
<td>Yes</td>
<td>0/16 (0)</td>
<td>0/16 (0)</td>
<td>1/16 (6.3)</td>
<td>10/16 (62.5)</td>
<td></td>
</tr>
</tbody>
</table>

1) Cefazolin + minocycline group versus minocycline group, P = 0.002.
was not different from that of the minocycline group in non-neutropenic mice. *V. vulnificus* infections were mainly found among patients with immunocompromising conditions, such as liver cirrhosis, diabetes, or adrenal insufficiency (4). To mimic the immunocompromised status, a well-established neutropenic model in mice is utilized in which intra-peritoneal administration of a large inoculum of bacteria is intended to represent severe infections. Under the conditions of a high bacterial inoculum (1.5 × 10⁸ CFU) and neutropenia, mice treated by minocycline and cefazolin in combination had a higher survival rate than that of mice treated with minocycline alone. No mouse treated by cefazolin alone in either the neutropenic or non-neutropenic group survived. This result differs from a previous in vitro time-kill study, in which cefazolin exhibited sustained inhibitory effects in normal inoculum.

A superior therapeutic efficacy of the combination of cefazolin and minocycline was observed in a neutropenic mouse model with *V. vulnificus* infection. These findings suggest that cefazolin combined with minocycline may be clinically effective in immunocompromised patients with invasive *V. vulnificus* infections. The interest in step-down policy of antimicrobial therapy has increased recently, due to the increasing prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae*. Further clinical investigations are required to verify the potential advantage of cefazolin in combination with minocycline.

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**REFERENCES**