Susceptibility of *Propionibacterium acnes* isolated from patients with acne vulgaris to zinc ascorbate and antibiotics

Katsuhiro Iinuma 1
Norihisa Noguchi 2
Hidemasa Nakaminami 2
Masanori Sasatsu 2
Setsuko Nishijima 3
Isami Tsuboi 1

1 BML General Laboratory, Matoba, Kawagoe, Saitama, 2 Department of Microbiology, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo, 3 Department of Dermatology, Nishijima Skin Clinic, Osaka, Japan

**Purpose:** The in vitro antimicrobial activity of ascorbic acid derivatives against *Propionibacterium acnes* was tested either alone or in combination with a variety of antimicrobial agents, and their fractional inhibitory concentration index was determined using checkerboard tests. The antimicrobial effectiveness of zinc ascorbate in the treatment of acne vulgaris, either alone or in combination with antibiotics such as clindamycin that are commonly used in Japan for the treatment of acne vulgaris, was therefore examined.

**Materials and methods:** The antimicrobial susceptibility of 41 strains of clindamycin-sensitive and/or clindamycin-resistant *P. acnes* isolated from acne vulgaris patients was tested, in comparison with a type strain of *P. acnes*.

**Results:** Zinc ascorbate showed antimicrobial activity against a type strain of *P. acnes* and its concentration (0.064%) was sufficiently lower than the normal dose (5%) of other ascorbic acid derivatives. Combinations of zinc ascorbate with clindamycin, erythromycin, and chloramphenicol showed an additive effect, and zinc ascorbate alone effectively inhibited the growth of all *P. acnes* including clindamycin-resistant strains.

**Conclusion:** The results provide novel evidence that the combination of zinc ascorbate and clindamycin is effective for acne vulgaris treatment.

**Keywords:** antimicrobial susceptibility, ascorbic acid derivatives, combination therapy, checkerboard test

**Introduction**

*Propionibacterium acnes* is an anaerobic, Gram-positive skin microbe that colonizes sebaceous glands and pilosebaceous follicles.1 This organism is considered to play a principal role in the development of acne vulgaris.1 Acne vulgaris is a chronic inflammatory disease characterized by typical inflammatory events, including the overproduction of sebum, abnormal desquamation of the sebaceous follicle epithelium, and *P. acnes* proliferation.1–3 Regarding the pathogenic factors for acne development and aggravation, ultraviolet irradiation and peroxidation of sebum lipids have been reported to activate inflammatory mediators such as reactive oxygen species.4

Ascorbic acid derivatives are some of the most widely used antioxidants for protecting the skin.5,6 The antioxidative effect of 5% sodium ascorbyl phosphate has demonstrated efficacy in acne vulgaris.7 However, it remains unclear whether ascorbic acid derivatives have antimicrobial activity against *P. acnes*.

Many topical and systemic treatments have been proposed for acne vulgaris.8 Clindamycin and erythromycin are the most frequently used agents against *P. acnes*.8,9 On the other hand, many studies have reported the emergence of *P. acnes* with high
level resistance to macrolides including clindamycin because long-term antibiotic therapy is commonly used to treat acne vulgaris.10–12 In Japan, a study conducted between 1994 and 1995 revealed that macrolide-resistant strains were found in only 4% (2/50) of P. acnes isolates from patients with acne vulgaris. However, they were found in 10% (5/48) of P. acnes isolates between 2006 and 2007, showing P. acnes resistance to macrolides has been increasing in Japan.8,9

Macrolide resistance in P. acnes is considered to be caused by mutation of the peptidyl transferase region in the domain V of 23S rRNA, and by the target site alteration with the 23S rRNA dimethylase that is encoded by erm(X).13 In addition, these mutations are associated with cross resistance to clindamycin.14 Therefore, the increasing prevalence of macrolide-resistant P. acnes, including clindamycin-resistant P. acnes, is a serious problem, because acne treatment by clindamycin is extremely difficult.

Recently, it has been reported that antibiotic combination enhances the therapeutic effect.15,16 In this study, the in vitro efficacy of ascorbic acid derivatives against P. acnes alone and in combination with clindamycin, erythromycin, chloramphenicol, minocycline, or levofloxacin was examined.

**Material and methods**

**Bacterial strains and drugs**

A total of 41 P. acnes strains used in this study were isolated from patients with acne vulgaris between 2006 and 2007 in Japan, and four strains of these were clindamycin-resistant P. acnes with mutation of the peptidyl transferase region in the domain V of 23S rRNA gene: A2058G, or A2059G.9 The patients were given topical antibiotics instead of systemic antibiotics. The samples were cultured on modified Gifu anaerobic medium (GAM) agar (Nissui Pharmaceutical Co, Tokyo, Japan) under anaerobic conditions at 35°C for 72 hours. P. acnes was identified by Api 20A (bioMérieux, Marcy l’Etoile, France).5 P. acnes JCM 6473 (ATCC 11828) strain was used as the quality control strain for antimicrobial susceptibility testing. Clindamycin, erythromycin, chloramphenicol, and minocycline were purchased from Sigma-Aldrich (Tokyo, Japan). All other chemicals used were of the highest analytical grade.

**Susceptibility tests**

Susceptibility testing was performed using microbroth dilution methods, according to the criteria of the Japanese Society of Chemotherapy.17,18 The samples were cultured in GAM broth (Nissui Pharmaceutical Co) and adjusted to a McFarland standard number 1. A dilute bacterial suspension was used to inoculate wells of a 96-well microplate, each well containing a different concentration of the drug being tested. Double dilutions of the drugs were prepared. The concentrations of the drugs in the GAM broth ranged from 0.06 to 128 µg/mL (antimicrobial agents) or 1.25 to 1280 µg/mL (ascorbic acid derivatives), and a final concentration of 10⁵ colony-forming units (CFU) of test bacteria per well was added to each dilution. The plates were incubated in an anaerobic gas-generating pouch (Anaero Pack System; Mitsubishi Gas Chemical Co, Tokyo, Japan) at 35°C for 48 hours. After the positive control lacking the antimicrobial agent demonstrated good growth, the minimum inhibitory concentration (MIC) for each antibiotic was defined as the lowest concentration of antibiotic required to inhibit bacterial growth, indicated by the absence of turbidity.

**Fractional inhibitory concentration (FIC) index**

The efficacy of a combination of zinc ascorbate and antimicrobial agents against P. acnes was determined by checkerboard tests using microbroth dilution methods.19 Fractional inhibitory concentration (FIC) indices were calculated using the following formula: FIC index = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone).20 An index of <0.5 was considered as synergism; that >1.0 but >0.5 as additive action; and that >2.0 as antagonism. The samples were adjusted to the McFarland standard number 1 and a final concentration of 10⁵ CFU/well of test bacteria. The MICs of the combinations were determined after incubation at 35°C for 48 hours.

**Results**

**The antibiotic susceptibility of P. acnes to ascorbic acid derivatives**

The antibiotic susceptibility of P. acnes JCM 6473 to ascorbic acid derivatives zinc ascorbate, magnesium ascorbyl phosphate, and sodium ascorbyl phosphate was investigated (Table 1). The MIC of zinc ascorbate was 640 µg/mL, whereas the MICs of ascorbic acid, magnesium ascorbyl phosphate, and sodium ascorbyl phosphate were ≥1280 µg/mL. The normal dose of ascorbic acid derivatives is 5% (50 mg/mL).7 These results indicate that zinc ascorbate sufficiently inhibits the growth of P. acnes in the normal dose. Thus, zinc ascorbate was used in further experiments.
Combined effect of zinc ascorbate and various antimicrobial agents against *Propionibacterium acnes*

To study the antimicrobial effects of combinations of zinc ascorbate and antimicrobial agents such as clindamycin, erythromycin, chloramphenicol, minocycline, and levofloxacin, the FIC index was determined by checkerboard tests (Table 2). In combinations of zinc ascorbate with clindamycin, erythromycin, or chloramphenicol, the MIC of zinc ascorbate reduced, and the FIC indices ranged from 0.625 to 0.75. The values exhibited an additive effect against *P. acnes* JCM 6473. Conversely, the FIC indices for the combinations of zinc ascorbate with minocycline or levofloxacin showed no effect.

Clindamycin is approved and commonly used in Japan for the treatment of acne vulgaris.8,9 Thus, the antimicrobial effect of zinc ascorbate combined with clindamycin for 37 strains of clindamycin-sensitive *P. acnes* isolated from patients with acne vulgaris was investigated. As shown in Table 3, the MIC range of zinc ascorbate was approximately twofold lower in combination with clindamycin compared with that of zinc ascorbate alone. The average FIC index was 0.84, exhibiting an additive effect.

### Table 1 Susceptibility of *Propionibacterium acnes* JCM 6473 to ascorbic acid derivatives

<table>
<thead>
<tr>
<th>Ascorbic acid derivatives</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>≥1280</td>
</tr>
<tr>
<td>Zinc ascorbate</td>
<td>640</td>
</tr>
<tr>
<td>Magnesium ascorbyl phosphate</td>
<td>≥1280</td>
</tr>
<tr>
<td>Sodium ascorbyl phosphate</td>
<td>≥1280</td>
</tr>
</tbody>
</table>

**Note:** The interaction was defined as synergistic if the FIC index was <0.5, additive if the FIC index was between 0.5 and 1.0, antagonistic if the FIC index ≥2.

**Abbreviations:** MIC, minimum inhibitory concentration (µg/mL); FIC, fractional inhibitory concentration.

### Combined effect of zinc ascorbate and various antimicrobial agents against clindamycin-resistant *P. acnes* isolated from patients with acne vulgaris

Clindamycin-resistant *P. acnes* with mutation of the peptidyl transferase region in the domain V of 23S rRNA has been increasing in Japan.9 The antimicrobial effect of zinc ascorbate combined with clindamycin, erythromycin, and chloramphenicol for four strains (PA001–PA004) of clindamycin-resistant *P. acnes* with a transition of adenine to guanine at the position of 2058 (A2058G: PA001–PA003) or 2059 (A2059G: PA004) was investigated. As shown in Table 4, zinc ascorbate was equally effective at inhibiting the growth of clindamycin-resistant *P. acnes* strains (MIC 640 µg/mL). In combinations of zinc ascorbate with clindamycin, erythromycin, and chloramphenicol, the MIC of zinc ascorbate reduced (MIC range: 160–320 µg/mL). In addition, the FIC index of PA004 strain with clindamycin-resistant *P. acnes* was 0.75, exhibiting an additive effect.

### Table 2 Combined effects of zinc ascorbate and antimicrobial agents against *Propionibacterium acnes* JCM 6473

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC (FIC index)</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinc ascorbate</td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Zinc ascorbate/Clindamycin</td>
<td>80/0.02 (0.63)</td>
<td>Additive</td>
</tr>
<tr>
<td>Zinc ascorbate/Erythromycin</td>
<td>160/0.06 (0.75)</td>
<td>Additive</td>
</tr>
<tr>
<td>Zinc ascorbate/Chloramphenicol</td>
<td>160/1 (0.75)</td>
<td>Additive</td>
</tr>
<tr>
<td>Zinc ascorbate/Minocycline</td>
<td>640/0.25 (2)</td>
<td>Antagonism</td>
</tr>
<tr>
<td>Zinc ascorbate/Levofloxacin</td>
<td>640/0.5 (2)</td>
<td>Antagonism</td>
</tr>
</tbody>
</table>

**Note:** The interaction was defined as synergistic if the FIC index was <0.5, additive if the FIC index was between 0.5 and 1.0, antagonistic if the FIC index ≥2.

**Abbreviations:** MIC, minimum inhibitory concentration (µg/mL); FIC, fractional inhibitory concentration.

### Discussion

In the microbroth dilution methods, according to the criteria of the Japanese Society of Chemotherapy, a twofold serial dilution series is used to determine the effectiveness of the test drug, with 1 µg/mL as the primary dilution.17,18 In this study, the concentrations of the drugs ranged from 0.06 to 128 µg/mL. However, ascorbic acid derivatives showed no effect in this concentration. It was confirmed that MIC of zinc was 1280 µg/mL (data not shown), which is similar to that reported by Fluhr et al.21 Therefore, the concentrations of ascorbic acid derivatives ranged from 1.25 to 1280 µg/mL.

Five percent of sodium ascorbyl phosphate has been reported to reduce acne lesions by 20.14% and 48.82% within 4 and 8 weeks, respectively.7 In addition, topical (1.2%) and systemic (30 mg) zinc have been reported to show efficacy in the treatment of acne vulgaris.21,22 In the present study, the MIC (640 µg/mL) of zinc ascorbate against *P. acnes* JCM 6473 corresponded to 0.064%, and the MIC value was lower than other ascorbic acid derivatives (MIC ≥1280 µg/mL). Although further experiments are needed to examine the drug kinetics and the stability of zinc ascorbate, it is expected that the calculating concentration of zinc ascorbate would be 2800 µg/mL when it is applied on the skin (4 mg/cm²) as an external preparation.23 These results suggest a possibility that zinc ascorbate (MIC...
Table 3 Combined effect of zinc ascorbate and clindamycin against 37 strains of Propionibacterium acnes isolated from patients with acne vulgaris

<table>
<thead>
<tr>
<th>Drug</th>
<th>MIC ranges (MIC₉₀)</th>
<th>FIC Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alone</td>
<td>Combined</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.13–2</td>
<td>0.06–1</td>
</tr>
<tr>
<td>Zinc ascorbate</td>
<td>160–640</td>
<td>80–320</td>
</tr>
</tbody>
</table>

Abbreviations: MIC, minimum inhibitory concentration (µg/mL); FIC, fractional inhibitory concentration; MIC₉₀, concentration of drugs which inhibited growth in 90% of the strains tested.

Table 4 Combined effect of zinc ascorbate and antimicrobial agents against four strains of macrolides-resistant Propionibacterium acnes isolated from patients with acne vulgaris

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC (FIC index)</th>
<th>AZn</th>
<th>CLDM</th>
<th>EM</th>
<th>CP</th>
<th>AZn/CLDM</th>
<th>AZn/EM</th>
<th>AZn/CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA001</td>
<td>640 ≥128</td>
<td>≥128</td>
<td>≥128</td>
<td>2</td>
<td>160/≥128 (NC)</td>
<td>160/≥128 (NC)</td>
<td>320/1 (1)</td>
<td></td>
</tr>
<tr>
<td>PA002</td>
<td>640 ≥128</td>
<td>≥128</td>
<td>≥128</td>
<td>2</td>
<td>320/≥128 (NC)</td>
<td>320/≥128 (NC)</td>
<td>320/1 (1)</td>
<td></td>
</tr>
<tr>
<td>PA003</td>
<td>640 ≥128</td>
<td>≥128</td>
<td>≥128</td>
<td>1</td>
<td>320/≥128 (NC)</td>
<td>160/≥128 (NC)</td>
<td>160/0.5 (0.75)</td>
<td></td>
</tr>
<tr>
<td>PA004</td>
<td>640 64</td>
<td>≥128</td>
<td>≥128</td>
<td>1</td>
<td>160/32 (0.75)</td>
<td>320/≥128 (NC)</td>
<td>160/0.5 (0.75)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: MIC, minimum inhibitory concentration (µg/mL); FIC, fractional inhibitory concentration; PA, Propionibacterium acnes; AZn, zinc ascorbate; CLDM, clindamycin; EM, erythromycin; CP, chloramphenicol; NC, not calculated.
Conclusion
The results of this study provide novel evidence that zinc ascorbate inhibits the growth of *P. acnes*, and its concentration (0.064%) is sufficiently lower than the normal dose (5%) of other ascorbic acid derivatives. In addition, zinc ascorbate shows an additive antimicrobial effect in vitro in combination with clindamycin, erythromycin, and chloramphenicol against clindamycin-sensitive and/or clindamycin-resistant *P. acnes*. These findings may provide novel insights into acne therapy. Recently, combination therapy with benzoyl peroxide and clindamycin has become common worldwide. Further experiments are needed to compare zinc ascorbate plus clindamycin and benzoyl peroxide plus clindamycin.

Disclosure
The authors report no conflicts of interest in this work.

References