Clinical, Cosmetic and Investigational Dermatology

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ORIGINAL RESEARCH

Zinc ascorbate has superoxide dismutase-like activity and in vitro antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli*

This article was published in the following Dove Press journal:
Clinical, Cosmetic and Investigational Dermatology
14 September 2012
Number of times this article has been viewed

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Background: Acne vulgaris is a common dermatological disease, and its pathogenesis is multifactorial.

Objective: We examined whether the ascorbic acid derivative zinc ascorbate has superoxide dismutase (SOD)-like activity. SOD is an enzyme that controls reactive oxygen species production. In addition, the in vitro antimicrobial activity of zinc ascorbate against the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli* was tested either alone or in combination with a variety of antimicrobial agents; their fractional inhibitory concentration index was determined using checkerboard tests.

Methods: The SOD-like activity was measured in comparison with other ascorbic acid derivatives (ascorbic acid, magnesium ascorbyl phosphate, and sodium ascorbyl phosphate) and zinc. The antimicrobial susceptibility of twelve strains each of *S. aureus* and *E. coli* isolated from patients with dermatological infections was tested, in comparison to a type strain of *S. aureus* and *E. coli*.

Results: Zinc ascorbate had significant ($P < 0.001$) SOD-like activity compared with other ascorbic acid derivatives and zinc. Moreover, it showed antimicrobial activity against a type strain of *S. aureus* and *E. coli*, and its concentration (0.064% and 0.128% for *S. aureus* and *E. coli*, respectively) was sufficiently lower than the normal dose (5%) of other ascorbic acid derivatives. Furthermore, combinations of zinc ascorbate with clindamycin, erythromycin, and imipenem against *S. aureus* (average fractional inhibitory concentration, 0.59–0.90), and with imipenem against *E. coli* (average fractional inhibitory concentration, 0.64) isolated from patients with dermatological infections showed an additive effect.

Conclusions: Our results provide novel evidence that zinc ascorbate may be effective for acne treatment.

Keywords: superoxide dismutase, reactive oxygen species, antimicrobial susceptibility, ascorbic acid derivatives, combination therapy

Introduction

Acne vulgaris is a common skin disorder affecting the pilosebaceous unit.[1] The pathogenesis of acne is attributed to multiple factors, such as increased sebum production, follicular hyperkeratinization, and proliferation of the Gram-positive bacterium *Propionibacterium acnes* within follicles.[1,2] Recently, reactive oxygen species (ROS) have been identified as inflammatory mediators in acne vulgaris. *P. acnes* infection causes the release of chemotactic factors leading to neutrophil accumulation, and ROS generated by the attracted neutrophils contribute to an inflammatory reaction, correlating with acne development and skin aggravation in acne vulgaris.[3]

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The control of ROS production is necessary for physiological cell function. Increased ROS are scavenged by superoxide dismutase (SOD).[4] SOD converts superoxide anion free radicals, detrimental to all living cells, to hydrogen peroxide and molecular oxygen.[4] Only a few studies on SOD in acne pathology have been conducted.[5,6] SOD activity in polymorphonuclear leukocytes has been reported to be significantly lower in acne patients than in a group of control patients. Therefore, drugs with SOD activity are considered useful for acne treatment.

Ascorbic acid derivatives are one of the most widely used antioxidants for protecting the skin.[7] The antioxidative effect of 5% sodium ascorbyl phosphate has demonstrated efficacy in acne vulgaris.[8] In addition, ascorbic acid derivatives conventionally have SOD-like activity.[9] However, several different ascorbic acid derivatives exist, and the differences in their effects remain unknown.

S. aureus and *E. coli* exist in the skin lesions of acne patients; they are associated with acne development in concert with *P. acnes*.[10–12] We recently reported that the ascorbic acid derivative zinc ascorbate inhibits the growth of *P. acnes* in vitro, and it may provide novel insights into acne therapy.[13] However, it remains unclear whether zinc ascorbate shows antimicrobial activity for other skin bacteria in addition to *P. acnes*.

In the present study, we examined the SOD-like activity of ascorbic acid derivatives. Furthermore, we examined the in vitro antimicrobial efficacy of zinc ascorbate against *S. aureus* and *E. coli* alone and in combination with various antimicrobial agents.

Materials and methods

Bacterial strains and drugs

The twelve *S. aureus* and twelve *E. coli* strains used in this study were isolated from patients with dermatological infections in Japan. The samples were cultured on modified trypticase soy agar containing 5% sheep blood (Becton Dickinson, Tokyo, Japan) under aerobic conditions at 35°C for 24 hours. *S. aureus* and *E. coli* were identified according to *Bergey's Manual of Determinative Bacteriology*.[14,15] *S. aureus* JCM 2874 (ATCC 29213) and *E. coli* JCM 5491 (ATCC 25922) were used as positive control strains for antimicrobial susceptibility testing. Clindamycin, erythromycin, and minocycline were purchased from Sigma-Aldrich (Tokyo, Japan). Ascorbic acid was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Magnesium ascorbyl phosphate and sodium ascorbyl phosphate were purchased from Showa Denko (Tokyo, Japan). All the other chemicals utilized in this study were of the highest analytical grade used.

Measurement of SOD-like activity

Ascorbic acid derivatives in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) were subjected to measurement of SOD-like activity using a SOD activity-detection kit (Wako), according to the manufacturer's instructions.[16,17] In addition, the ascorbic acid derivatives themselves are a reaction-interfering substance using this kit, because they diluted to a concentration that did not give an error to measured value, and SOD-like activity was computed from the dilution rate. The principle of this kit was as follows. The superoxide anion radical is formed from xanthine by the action of xanthine oxidase contained in the enzyme solution. The superoxide anion radical thus produced reduces nitroblue tetrazolium and forms diformazan. When SOD is contained in a sample, partial superoxide anion radical is dismutated into hydrogen peroxide and oxygen, and the production of diformazan is markedly inhibited by competing for the superoxide anion radical. SOD-like activity of the sample is determined by measuring the inhibition rate of diformazan production against a blind sample of non-SOD-like activity.

Susceptibility tests

Susceptibility testing was performed using microbroth dilution methods, according to the criteria of the Japanese Society of Chemotherapy.[18] Bacterial samples were cultured in Mueller Hinton broth (Becton Dickinson) and adjusted to the 0.5 McFarland standard. A dilute bacterial suspension was used to inoculate the wells of a 96-well microplate, with each well containing a different concentration of the drug being tested. We prepared double dilutions of the drugs; the concentrations of the drugs in the Mueller Hinton broth ranged from 0.06 to 128 μg/mL (for antimicrobial agents) or 1.25 to 1280 μg/mL (for ascorbic acid derivatives). A final concentration of 10^5 colony-forming units of test bacteria per well was added to each dilution. The plates were incubated at 35°C for 24 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 the antibiotic required to inhibit bacterial growth, indicated by the absence of turbidity.

Fractional inhibitory concentration index

The efficacy of the combination of zinc ascorbate and antimicrobial agents such as clindamycin, erythromycin,

imipenem, minocycline, and levofloxacin against twelve strains each of *S. aureus* and *E. coli* isolated from patients with dermatological infections was determined by checkerboard tests using microbroth dilution methods.[13,19] Fractional inhibitory concentration (FIC) indices were calculated using the following formula: FIC index = (MIC of zinc ascorbate in combination with antimicrobial agent/MIC of zinc ascorbate alone) + (MIC of antimicrobial agent in combination with zinc ascorbate/MIC of antimicrobial agent alone).[13,20] An FIC index less than 0.5 indicated synergism; less than 1.0 but greater than 0.5 indicated additive action; less than 2.0 but greater than 1.0 indicated indifference; and greater than 2.0 indicated antagonism. The samples were adjusted to the 0.5 McFarland standard and a final concentration of 10^5 colony-forming units/well of test bacteria. MICs of the drug combinations were determined after incubation at 35°C for 24 hours.

Statistical analysis

Data are presented as means ± standard deviation and were analyzed by one-way analysis of variance and the Fisher test for multiple comparisons. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Ascorbic acid derivatives exhibit SOD-like activity

To clarify the difference in the effect of various ascorbic acid derivatives, we examined their SOD-like activity. SOD is an enzyme that participates in the removal of ROS. As shown in Figure 1A, zinc ascorbate was found to have significant ($P < 0.001$) SOD-like activity compared with other ascorbic acid derivatives and zinc. In addition, it was found that zinc ascorbate increased the level of SOD-like activity in a dose-dependent manner (Figure 1B). As shown in Table 1, when referring to equimolar levels (25 μM), zinc ascorbate, ascorbic acid, and zinc showed SOD-like activity. However, magnesium ascorbyl phosphate and sodium ascorbyl phosphate showed little or no SOD-like activity.

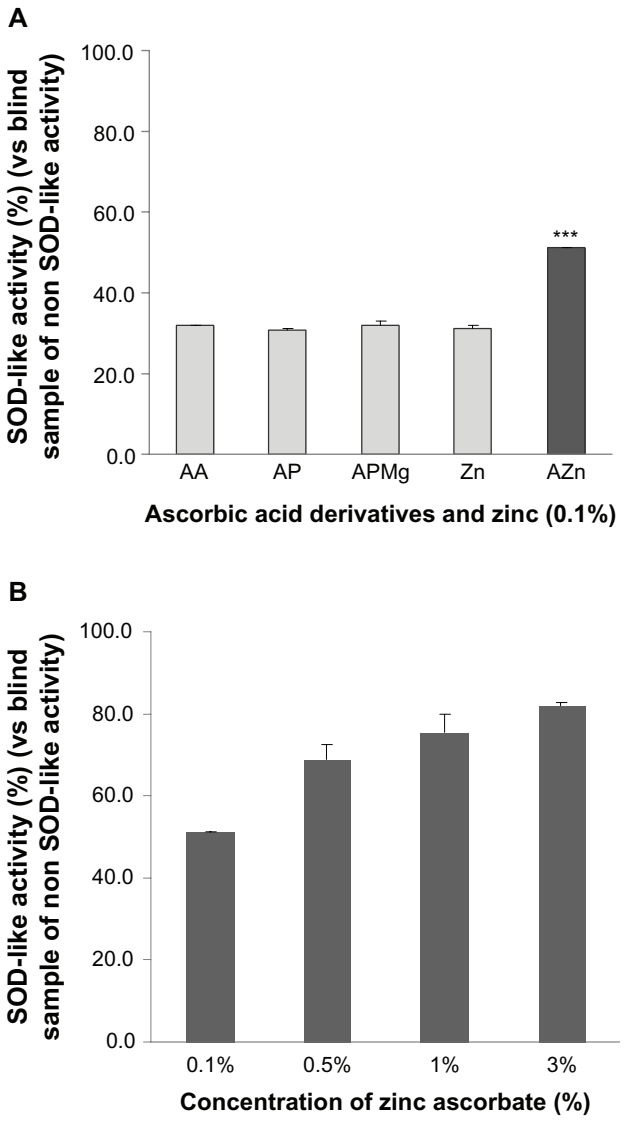

Figure 1 Superoxide dismutase (SOD)-like activity of ascorbic acid derivatives and zinc. (**A**) Ascorbic acid derivatives and zinc (0.1%) and (**B**) zinc ascorbate (AZn) (0.1%–3%) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) were subjected to the measurement of SOD-like activity (%), as described in the text. Data are indicated as mean ± standard deviation of triplicate assays.
Notes: ***Significantly different from ascorbic acid (AA), magnesium ascorbyl phosphate (APMg), sodium ascorbyl phosphate (AP), and zinc (Zn) ($P < 0.001$, respectively).

Antibiotic susceptibility of *S. aureus* and *E. coli* to zinc ascorbate

The antibiotic susceptibility of *S. aureus* JCM 2874 and *E. coli* JCM 5491 to zinc ascorbate was examined. As shown in Table 2, MIC of zinc ascorbate was 640 μg/mL against *S. aureus* and 1280 μg/mL against *E. coli*, whereas that of other ascorbic acid derivatives (ascorbic acid, magnesium ascorbyl phosphate, and sodium ascorbyl phosphate) was >1280 μg/mL (data not shown). The normal dose of ascorbic acid derivatives for acne treatment is 5% (50 mg/mL).[8] Therefore, these results indicate that zinc ascorbate sufficiently inhibits the growth of *S. aureus* and *E. coli* in the normal dose.

Combined effect of zinc ascorbate and various antimicrobial agents against *S. aureus* and *E. coli*

In Japan, orally administered macrolides, β-lactams, tetracycline, and fluoroquinolones are approved for treating

Table 1 Superoxide dismutase (SOD)-like activity of 25 μM ascorbic acid derivatives and zinc

Drug	SOD-like activity (%)
Ascorbic acid	18.4 ± 1.7
Sodium ascorbyl phosphate	ND
Magnesium ascorbyl phosphate	1.4 ± 0.6
Zinc	9.7 ± 0.6
Zinc ascorbate	26.7 ± 0.1

Notes: Ascorbic acid derivatives and zinc (25 μM) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2) were subjected to measurement of SOD-like activity (%), as described in the text. Data are indicated as mean ± standard deviation of triplicate assays.
Abbreviation: ND, not detected.

patients with acne vulgaris.[21] Of the topical antibiotics, clindamycin and nadifloxacin are approved and commonly used in Japan for acne treatment.[21] To study the combined effect of zinc ascorbate and antimicrobial agents against twelve clinical strains each of *S. aureus* and *E. coli*, the FIC index was determined by checkerboard tests. As shown in Table 3, in combinations of zinc ascorbate with clindamycin, erythromycin, and imipenem, the average FIC indices ranged from 0.59 to 0.90, and the values exhibited an additive effect against *S. aureus*. In addition, in combinations of zinc ascorbate with impenem, the average FIC index was 0.64, and the values exhibited an additive effect against *E. coli*. On the other hand, it is possible that neither clindamycin nor erythromycin show antimicrobial activity against *E. coli*,and minocycline and levofloxacin chelate with metal ions, resulting in reduced antimicrobial activity.[22–24]

Discussion

ROS generated by neutrophils are closely correlated with the pathogenesis of a variety of inflammatory skin disease, eg, acne vulgaris, Behçet's disease, and psoriasis.[4] Akamatsu et al reported that patients with acne inflammation had a significantly increased level (43%) of ROS produced by neutrophils compared with healthy controls.[3] SOD is an antioxidant enzyme that has a role in the defense against ROS.[4] SOD activity is significantly lower in acne patients (0.17 ± 0.005 IU/mg protein) than in controls (0.31 ± 0.007 IU/mg protein).[5] Therefore, drugs with SOD activity have been considered useful in acne treatment.

Table 2 Susceptibility of *Staphylococcus aureus* JCM 2874 and *Escherichia coli* JCM 5491 to zinc ascorbate

Bacterial species	MIC
S. aureus	640
E. coli	1280

Abbreviation: MIC, minimum inhibitory concentration (μg/mL).

Several ascorbic acid derivatives have been described, eg, ascorbic acid, zinc ascorbate, magnesium ascorbyl phosphate, and sodium ascorbyl phosphate. In the present study, zinc ascorbate had significant ($P < 0.001$) SOD-like activity compared with other ascorbic acid derivatives. This result suggests that zinc ascorbate may suppress ROS production, rather than other ascorbic acid derivatives and zinc. There was no difference between ascorbyl phosphate, magnesium ascorbyl phosphate, and ascorbic acid, because we examined these using w/w (%) solution. When referred to as equimolar levels (25 μM), zinc ascorbate, ascorbic acid, and zinc showed SOD-like activity. On the other hand, magnesium ascorbyl phosphate and sodium ascorbyl phosphate showed little or no SOD-like activity. However, we confirmed that sodium ascorbyl phosphate increases the level of SOD-like activity in a dose-dependent manner (25 μM, not detected; 50 μM, 3.6%; 75 μM, 9.0%). Ascorbic acid and zinc are known to scavenge superoxide anion radical generated by the xanthine–xanthine oxidase system.[25,26] Therefore, it is thought that zinc ascorbate showed SOD-like activity rather than other ascorbic acid derivatives and zinc.

Recently, we reported that zinc ascorbate inhibits the growth of *P. acnes* (MIC, 640 μg/mL).[13] However, it remains unclear whether zinc ascorbate has antimicrobial activity against not only *P. acnes* but also any other bacterium. In the present study, MIC of zinc ascorbate against *S. aureus* JCM 2874 (MIC, 640 μg/mL) and *E. coli* JCM 5491 (MIC, 1280 μg/mL) are lower than those of other ascorbic acid derivatives (MIC, >1280 μg/mL). In addition, we confirmed that MICs of zinc against *S. aureus* and *E. coli* were 1280 μg/mL and >1280 μg/mL, respectively (data not shown). It has been reported that antimicrobial activity of ascorbic acid derivatives on bacterium differs by its species and strains.[27] The Gram-positive bacterium *S. aureus* and Gram-negative bacterium *E. coli* exist as resident microflora on human skin and are associated with acne development in concert with *P. acnes*.[10–12] Therefore, zinc ascorbate may sufficiently inhibit the growth of *S. aureus* and *E. coli*, which participate in acne development in the concentration that is lower than other ascorbic acid derivatives and zinc, similar to its effect on *P. acnes*.[13] These results provide novel evidence that zinc ascorbate will be useful for treating acne vulgaris.

Ascorbic acid derivatives enhance an antimicrobial activity by combined effect of metal ion, but its activity changes with the kind of metal ion.[27,28] Zinc and its salts exhibit well-known antibacterial activity.[29] On the other hand, ascorbic acid derivatives show a prevention activity by combined effect of the metal chelaters, eg, citrate and

Table 3 Combined effects of zinc ascorbate and antimicrobial agents on the twelve clinical strains each of *Staphylococcus aureus* and *Escherichia coli*

Drug combination	*S. aureus*		*E. coli*	
	FIC range (average)	Interaction	FIC range (average)	Interaction
Zinc ascorbate + clindamycin	0.50–1 (0.79)	Additive	2 (2)	Indifference
Zinc ascorbate + erythromycin	0.75–1 (0.90)	Additive	2 (2)	Indifference
Zinc ascorbate + imipenem	0.28–0.75 (0.59)	Additive	0.38–0.75 (0.64)	Additive
Zinc ascorbate + minocycline	2 (2)	Indifference	2 (2)	Indifference
Zinc ascorbate + levofloxacin	2 (2)	Indifference	2 (2)	Indifference

Notes: The interaction was defined as synergistic if FIC index was less than 0.5; it was defined as additive if FIC index was between 0.5 and 1.0; it was defined as indifferent if FIC index was between 1.0 and 2.0; and it was defined as antagonistic if FIC index was >2.
Abbreviation: FIC, fractional inhibitory concentration.

ethylenediamine-N,N,N′,N′-tetraacetic acid.[28] Therefore, we hypothesize that zinc ascorbate shows an antimicrobial activity stronger than zinc citrate. Further experiments are needed to compare zinc ascorbate with other zinc compounds.

Combined antibiotic treatments have been reported to enhance therapeutic effect.[30] In addition, combined therapy is useful for preventing the emergence of antibiotic-resistant strains of *P. acnes*.[30] Clindamycin is approved and commonly used in Japan for acne treatment.[21] Recently, we suggested that the combination of zinc ascorbate and clindamycin would be useful to prevent the emergence of clindamycin-resistant *P. acnes* strains and treat acne vulgaris.[13] In the present study, the combination of zinc ascorbate with clindamycin against *S. aureus* was found to exhibit an additive effect (average FIC, 0.79), whereas it was found to exhibit an indifference effect against *E. coli*. Gram-negative bacteria–derived lipopolysaccharide induces neutrophils, and ROS production is enhanced.[31] In addition, some drugs used in acne treatment, such as tetracycline and macrolide, show the ability to suppress an inflammatory reaction mediated by ROS in addition to their antibacterial activity.[3,32] Furthermore, clindamycin scavenges hydroxyl radical, whereas it does not scavenge superoxide anion radical.[33] In the present study, zinc ascorbate was found to have SOD-like activity, and it is possible that this compound can suppress ROS production. Therefore, the combination of zinc ascorbate and clindamycin against *E. coli* may be useful for enhancing the suppression of ROS production. Further experiments are needed to clarify the mechanism of zinc ascorbate activity and the combined effect of zinc ascorbate and clindamycin.

In conclusion, our results provide novel evidence that zinc ascorbate dose-dependently increases the level of SOD-like activity and inhibits the growth of *S. aureus* (MIC, 640 μg/mL) and *E. coli* (MIC, 1280 μg/mL). Moreover, the combination of zinc ascorbate and clindamycin may be useful for treating acne vulgaris in vitro. To show that zinc ascorbate is useful as an antiacne agent, further experiments are needed to clarify the effectiveness in decreasing sebum production and follicular hyperkeratinization in the pathogenesis of acne.

Disclosure

The authors report no conflicts of interest in this work.

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