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Antiviral Activity of Ascorbic Acid Against Herpes Simplex Virus

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In order to explore the potential of ascorbic acid supplementation for the prevention and treatment of herpes simplex viral diseases, plaque reduction assays were performed. Ascorbic acid as well as copper chloride/ferric chloride were added to wells containing Vero cells infected with herpes simplex virus type 1 (HSV-1), and the infectivity of HSV-1 was determined. Since copper and iron are major transition metals in human plasma, near the normal human plasma concentrations of them were used for experiments. When Cu(II) and Fe(III) were applied, there were no significant differences between virus control and Cu(II)/Fe(III)-treated groups. But, when appropriate concentrations of ascorbic acid were added to wells, meaningful differences between control and ascorbate-treated groups were found. In the presence of Cu(II)/Fe(III) at 5.8/3.7 μM, 72-h treatment with ascorbate at 50 μM reduced HSV-1 infections to 10.77%±4.25% ($P < 0.001$) and 500 μM did to 3.06%±1.62% ($P < 0.001$). Moreover, the cytotoxicities for Vero cells at those concentrations were insignificant ($P > 0.05$). Current recommended dietary allowance (RDA) of ascorbic acid is 60 mg/day, and the oral intake of 60 mg/day of ascorbic acid yields plasma ascorbic acid at 45 to 58 μM in a healthy adult man. Therefore, the results of this study suggest that the maintenance of appropriate level (more than 50 μM) of ascorbic acid in human plasma by appropriate amount (more than the RDA) of ascorbic acid supplementation may be helpful for the prevention and treatment of diseases caused by HSV-1 in an adult man. In addition, this study also suggests that ascorbic acid may be useful for the prophylaxis of fatal HSV-1 infections in neonates and the prevention of HSV-1 reactivation in immunocompromised hosts.

Key Words: Ascorbic acid, Herpes simplex virus type 1, Antiviral activity

INTRODUCTION

Ascorbic acid is currently known as a potent water-soluble antioxidant (10,11) and an immune modulator (19). In addition, its deleterious effect on mammalian cells (4,5) and antiviral activity (15,16,29) have been investigated based on toxicity of it.

Ascorbic acid can interact with redox active transition metal ions, such as iron and copper ion. It maintains the active center metal ions of hydroxylase and oxygenase in a reduced state for optimal enzyme activity. Deficiency of ascorbic acid induces scurvy, resulting from decreased activity of these enzymes (21).

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Paradoxically, the reduction of transition metal ions by ascorbic acid could also have a deleterious effect on mammalian cells (3,14). Ascorbic acid can produce hydroxyl radicals or lipid alkoxyl radicals by a reaction of the reduced metal ions with hydrogen peroxide or lipid hydroperoxides. This Fenton reaction occurs readily *in vitro*, but *in vivo* relevance has been a matter of some controversy (4). The concentrations of free metal ions have been thought to be very low because of their sequestration by metal binding proteins such as ferritin, transferrin and ceruloplasmin (13). Hence, some researchers have thought that there is no Fenton reaction *in vivo* partly due to the low concentrations of free transition metal ions (4). During tissue injury, however, bound metal ions may be released and could interact with ascorbic acid (14).

Cupric ion (Cu(II)), one of the dominant transition metal ions in human plasma, binds specifically to DNA favoring guanosine residues. Then, it is reduced and can react with hydrogen peroxide or lipid hydroperoxides producing hydroxyl radicals or lipid alkoxyl radicals that can cause oxidative damage resulting in DNA single-strand breaks and base modifications (6, 9,18,29,30). In addition, Cupric ion has been shown to inactivate several types of viruses, including members of herpesvirus family (27). This inactivation more readily occurred when various reducing agents, such as ascorbic acid and cysteine, were present. The order of reducing agent enhancement on copper-mediated HSV-1 inactivation was similar to those previously observed for DNA damage mediated by copper and other transition metals. Thus, HSV-1 may be inactivated by a mechanism paralleling that observed in copper-mediated DNA damage (28).

Many researchers have reported that the vitamin C-containing topical agents for recurrent mucocutaneous herpes (recurrent herpes labialis) showed significant therapeutic effect (15,16). Virus culture yielded HSV-1 less frequently in the active treatment group. Furthermore, a previous report (28) revealed that when an appropriate concentration of ascorbic acid was used in the presence of near the normal total human plasma concentration of copper, inactivation of herpes simplex virus type 1 (HSV-1) occurred. Its results suggest that ascorbic acid supplementation may have antiviral activity *in vivo*.

Based on the knowledge described above, *in vitro* experiments were performed to explore the potential of ascorbic acid supplementation for the prevention and treatment of herpes simplex viral diseases. Ascorbic acid as well as transition metals added to wells containing Vero cells infected with HSV-1, and then plaque reduction assays were performed. Since iron and copper are major transition metals in human plasma, near the normal plasma concentrations of them were used. For estimating cytotoxicity of added chemicals, neutral red assays were performed. The antiviral activity of acycloguanosine (acyclovir) was determined to compare the effect of ascorbic acid with that of established antiviral agent.

MATERIALS AND METHODS

Cell line. Vero cells (African green monkey kidney fibroblasts), obtained from Korean Cell Line Bank (KCLB; Seoul), were maintained in growth medium at 37℃ in 5% CO_2 in air. Eagle's minimum essential medium (EMEM; GIBCO BRL, Grand Island, NY, USA) was used as growth medium. It was supplemented with 10% fetal bovine serum (FBS; GIBCO BRL), 100 U of penicillin G sodium per ml, 100 μg of streptomycin sulfate per ml and 0.25 μg of amphotericin B per ml (GIBCO BRL). For subculture, cells were dissociated with 0.1% trypsin/0.04% EDTA (GIBCO BRL).

Virus. Herpes simplex virus type 1 (HSV-1), F strain, was obtained from National Institute of Health, Korea (KNIH; Seoul) and stored at

-70℃ in aliquots in serum-free EMEM after growth on Vero cells.

Chemicals. L-Ascorbic acid sodium salt, cupric chloride ($CuCl_2 \cdot 2H_2O$), ferric chloride ($FeCl_3 \cdot 6H_2O$) and acycloguanosine were dissolved in distilled water. They were filtered with 0.2-μm-pore-size syringe filters (Acrodisc; Gelman Sciences, Ann Arbor, MI, USA) and stored at 4℃. 100 g of L-ascorbic acid sodium salt per liter, 1 g of cupric chloride per liter, 1 g of ferric chloride per liter and 0.1 g of acycloguanosine per liter stock solutions were prepared. Each stock was diluted in sterile distilled water immediately before each experiment. Neutral red (NR) stock (4 mg/ml) was prepared in distilled water, protected from light with foil, and stored at 4℃.

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Neutral red cell viability assay. For toxicity testing in cell culture, neutral red (NR) assays were performed. 96-well plates were fed with 4×10^3 cells in 0.2 ml of growth medium (EMEM with 10% FBS and antimicrobial agents) per well, and incubated at 37℃ in 5% CO_2 in air for 48 h. Thereafter, growth medium was replaced with treatment medium (EMEM with 2.5% FBS, antimicrobial agents, and various concentrations of the test chemicals). After 72-h incubation with treatment medium, the cytotoxicity was measured as described previously (2). Growth medium containing 40 μg of NR per ml was prepared and prewarmed at 37℃ overnight. Fine crystals were removed by centrifugation (700 × g, 5 min) after prewarming. The treatment medium in each well of 96-well plates was removed, and 0.2 ml of NR-containing medium was added to each well. Thereafter, the plates were incubated at 37℃ in 5% CO_2 in air for 3 h. Wells were rapidly rinsed and fixed with 0.2 ml per well of 0.5% formalin containing 1% $CaCl_2$ after incubation. 0.2 ml of 1% glacial acetic acid in 50% ethanol was added to each well and left for 15 min with shaking to release NR into supernatant. Absorbance at 540 nm was read with a microtiter plate reader (Emax; Molecular Devices Corporation, Menlo Park, CA, USA) after shaking. Results were expressed as percent of untreated control.

Plaque reduction assay. To test the antiviral activity of the chemicals, plaque reduction assays were performed as described previously (17), with modifications. Cultures of confluent Vero cells were prepared in 24-well plates, and the chemicals were diluted appropriately. HSV-1 seed stock was diluted to inoculate with 30-60 PFU per well, and the inoculated plates were incubated for 1 h. Dilution series of chemicals were prepared in overlay medium. The cell sheets were re-fed with 1 ml of overlay medium containing various dilutions of the chemicals per well after 1-h incubation for virus adsorption. Samples were duplicated for each dilution of the chemicals. After 72-h incubation, the plates were fixed, stained and counted. Results were expressed as percent of untreated control.

The concentrations of free copper and iron ions in human plasma are very low compared with the total concentrations of them. So, to determine the effect of copper and iron, Cu(II)/Fe(III) at 0.58/0.37, 5.8/3.7 and 58/37 μM were added to wells because 0.58/0.37 and 5.8/3.7 μM were below half of the normal total concentrations of copper/iron in human plasma and 58/37 μM were above the total concentrations of copper/iron.

To estimate the antiviral effect of ascorbic acid in the presence of transient metals, L-ascorbic acid sodium salt at 5, 50, 500 and 5,000 μM were mixed with various concentrations of Cu(II)/Fe(III) and added to wells because about 50 μmol of ascorbate per liter in human plasma is the yield of the oral intake of 60 mg/day of ascorbic acid (current RDA) in a healthy adult man.

The known dose of acycloguanosine inhibiting 50% of HSV-1 infections (ID_{50}) was 0.1-1.0

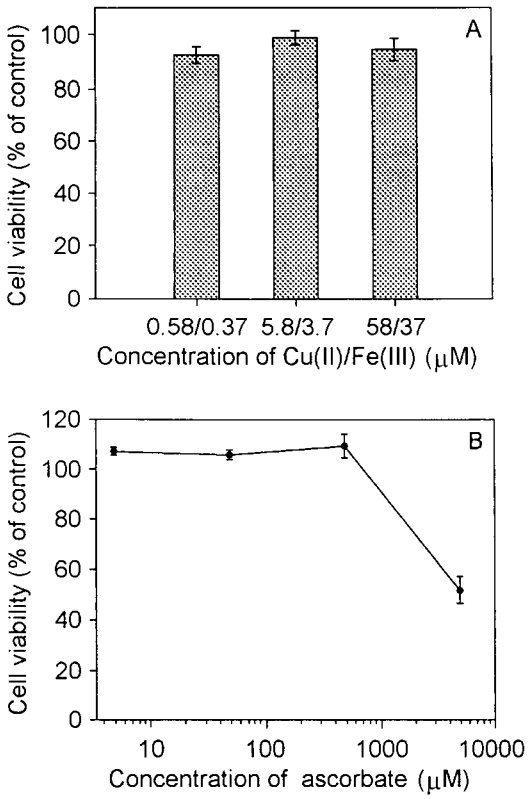

Figure 1. Cytotoxicity for Vero cells. Neutral red assays were conducted after treatment with Cu(II)/Fe(III) (a) or with ascorbate at 5 to 5,000 μM in the presence of Cu(II)/Fe(III) at 5.8/3.7 μM (b) for 72 h at 37℃ in 5% CO_2 in air. The viability of Vero cells was measured by reading absorbance at 540 nm and presented as percent of untreated control. Means±SEM from three experiments performed in duplicate are presented.

μM, and so 0.04, 0.4 and 4 μmol of acycloguanosine per liter were added to wells in order to determine antiviral activity of it.

Statistical analysis. Presented data are means ±standard errors of means (SEM) of three experiments. For estimating the significance of differences between control and test samples, Wilcoxon rank sum tests were performed.

RESULTS

Cytotoxicity for Vero cells. At 0.58/0.37, 5.8/3.7 and 58/37 μM, Cu(II)/Fe(III) showed no evidence of toxicity for Vero cells after 72-h

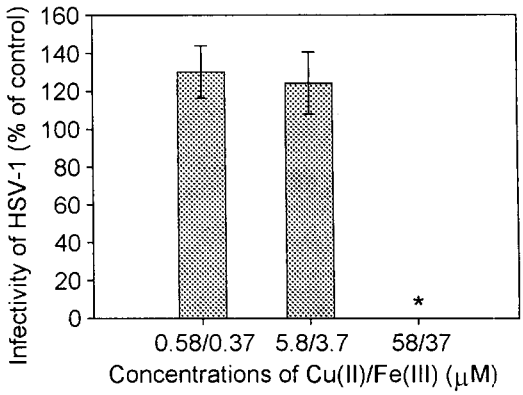

Figure 2. Effect of copper and iron on HSV-1. Cu(II)/Fe(III) at 0.58/0.37, 5.8/3.7 and 58/37 μM were used for plaque reduction assay. No plaques were found on the wells containing Cu(II)/Fe(III) at 58/37 μM after 72-h treatment at 37℃ in 5% CO_2 in air (*). Means±SEM from three experiments performed in duplicate are presented.

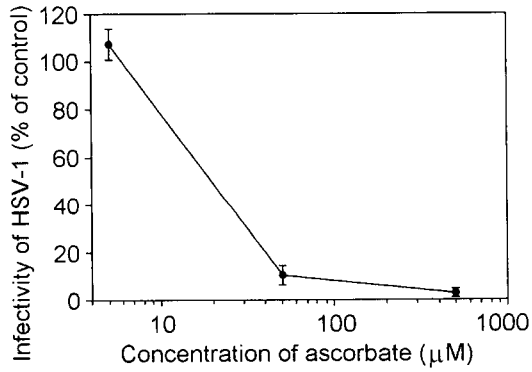

Figure 3. Antiviral effect of ascorbic acid against HSV-1 in the presence of copper and iron. 5 to 5,000 μmol of ascorbate per liter with 5.8/3.7 μmol of Cu(II)/Fe(III) per liter were added to the wells containing Vero cells infected with HSV-1. Plaques were counted after 72-h treatment at 37℃ in 5% CO_2 in air. Ascorbate at 5,000 μM disrupted Vero cell monolayer, and so plaque counting was impossible. Means ±SEM from three experiments performed in duplicate are presented.

treatment (Fig. 1a). With Cu(II)/Fe(III) at 5.8/3.7 μM, the cytotoxicities of 5, 50 and 500 μmol of ascorbate per liter were insignificant ($P > 0.05$). After the addition of 5,000 μmol of ascorbate per liter, however, the cytotoxicity for Vero cells was significant in the presence of Cu(II)/Fe(III) at 5.8/3.7 μM ($P < 0.001$) (Fig. 1b).

Table 1. Antiviral effect of ascorbic acid and that of acycloguanosine

Inhibitors (μM)	Infectivity of HSV-1 (% of control)[a]
Ascorbate	
+Cu(II)/Fe(III) (5.8/3.7)	
5	107.17±6.59
50	10.77±4.25[b]
500	3.06±1.62[b]
5,000	ND[c]
Acycloguanosine	
0.04	118.55±25.61
0.4	0[b,d]
4	0[b,d]

[a] The infectivity of HSV-1 was measured by the quantitation of plaque formation and presented as percent of untreated control.
[b] Statistically significant ($P < 0.001$).
[c] Not determined. Cell layer was disrupted, and so plaque counting was impossible.
[d] No plaques were found.
Means±SEM from three experiments performed in duplicate are presented.

Effect of copper and iron. There were no significant differences between virus control and test samples when Cu(II)/Fe(III) at 0.58/0.37 μM or at 5.8/3.7 μM was added to wells ($P > 0.05$). However, Cu(II)/Fe(III) at 58/37 μM completely inhibited HSV-1 infections as measured by the quantitation of plaque formation ($P < 0.001$) (Fig. 2).

Antiviral effect of ascorbic acid against HSV-1 in the presence of transient metals. In the presence of Cu(II)/Fe(III) at 5.8/3.7 μM, ascorbate at 50 and 500 μM inhibited HSV-1 infections significantly. Ascorbate at 5 μM did not inhibit HSV-1 infections, but 50 and 500 μM reduced HSV-1 infections significantly after 72-h treatment (Table 1, Fig. 3). Cu(II)/Fe(III) at 58/37 μM and/or ascorbate at 5,000 μM disrupted Vero cell monolayer, and so plaque counting was impossible.

The antiviral activity of acycloguanosine was determined to compare the effect of ascorbic acid with that of an established antiviral agent. After 72-h treatment, acycloguanosine at 0.4 μM completely inhibited HSV-1 infections while no antiviral activity was seen at 0.04 μM (Table 1). 0.04 (M of acycloguanosine was added to ascorbate/Cu(II)/Fe(III) mixture to investigate synergism between acycloguanosine and ascorbate, but there was no significant difference between the acycloguanosine/ascorbate-treated group and the ascorbate-treated group (data not shown).

DISCUSSION

The experiments described here were designed and performed to explore the potential of ascorbic acid supplementation for the prevention and treatment of herpes simplex viral diseases. And the results from these experiments showed significant differences between control and ascorbate-treated groups.

Unexpectedly, there was complete inhibition of HSV-1 infections after 72-h treatment with Cu(II)/Fe(III) at 58/37 μM, while there was no inhibition at 0.58/0.37 and 5.8/3.7 μM. Moreover, the cytotoxicity for Vero cells was insignificant at that concentration. With any concentrations of ascorbic acid used in this study, however, Cu(II)/Fe(III) at 58/37 μM disrupted Vero cell monolayer. Therefore, more than the normal total concentrations of Cu(II) and Fe(III) may be harmful rather than useful under physiological conditions that various reducing agents and transition metals coexist.

In the presence of Cu(II)/Fe(III) at 5.8/3.7 μM, ascorbate at 50 and 500 μM showed effective antiviral activity after treatment for 72 h. HSV-1 infections were inhibited significantly, and there was no evidence of cytotoxicity. It suggests that if more than 50 μmol of ascorbate per liter is maintained in human plasma, effective antiviral activity may be achieved *in vivo*.

Current recommended dietary allowance (RDA)

of ascorbic acid is 60 mg/day, which is the minimum dose to prevent scurvy (22). And the oral intake of 60 mg/day of ascorbic acid yields plasma ascorbate at 45 to 58 μM in a healthy adult man (8). Thus, the results described above suggest that the maintenance of appropriate level (more than 50 μM) of ascorbic acid in human plasma by an appropriate amount (more than the RDA) of ascorbic acid supplementation may be helpful for the prevention and treatment of diseases caused by HSV-1 in an adult man. Moreover, the results also suggest that ascorbic acid may be useful for the prophylaxis of fatal HSV-1 infections in neonates and the prevention of HSV-1 reactivation in immunocompromised hosts because these *in vitro* experiments were performed in immunodeficient state.

The experiments described here were performed *in vitro*. Therefore, there would be some limitations to apply the results of this study to physiological conditions. The most important question is whether the inhibition of HSV-1 infections also occurs under physiological conditions or not. Previously reported data on ascorbic acid and oxidative DNA damage on mammalian cells are inconsistent and conflicting (4). In the presence of transition metals, there was oxidative DNA damage by ascorbate *in vitro* (6,9,17). Ascorbate, however, served as an antioxidant *in vitro* in the absence of transition metals (7,9,24,31), although there were a few exceptions (1,12). Moreover, in spite of a few exceptions (25,26), many reports showed that ascorbic acid did not act as a pro-oxidant on mammalian cells under physiological conditions (4,12,20,23).

With the information mentioned above, one may come to an immediate conclusion that oxidative herpes simplex viral DNA damage does not occur *in vivo*. However, herpes viral DNA may differ from mammalian cellular DNA. The results of this study demonstrated that HSV-1 infections were inhibited significantly after the treatment with ascorbate, whereas Vero cells showed no significant cytotoxicity after that treatment. It may be ascribed to the fact that mammalian cells are readily repaired by various repairing systems in the cells but HSV-1 is not (28). Hence, it is supposed that herpes simplex viral DNA may be damaged significantly by ascorbate even *in vivo*.

In conclusion, this study suggests that ascorbic acid supplementation for the prevention and treatment of herpes simplex viral diseases may be of great value and have no hazardous effects to the hosts. To elucidate antiviral activity of ascorbic acid under physiological conditions, more studies, such as animal experiments and clinical trials, are needed.

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