Effect of High Concentration of Ascorbate on Catalase Activity in Cultured Cells and Tissues of Guinea Pigs

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Summary This study showed that the inhibition of cell growth in 3T6, induced by the supplementation of ascorbate at 0.5 mM in cultured medium, was prevented by the addition of catalase in the range of 25–750 units/mL regardless of the degree of activity. However, cytotoxicity induced by concentrations of more than 2 mM ascorbate could not be prevented even when a high level of catalase (5,000 units/mL) was added to the medium. Catalase activity in medium supplemented with ascorbate decreased with incubation time; the higher the concentration of ascorbate, the greater the decrease in catalase activity. These results indicate that even though catalase is present at a high concentration in the medium, it cannot prevent cytotoxicity by a high concentration of ascorbate because the oxygen radical derived from ascorbate inhibits its activity. We therefore investigated whether the inhibition of catalase activity by ascorbate could be observed in animal tissues. The catalase activity in the tissues of guinea pigs 6 h after the administration of ascorbate was lower than that in non-administered animals. When guinea pigs were fed diets containing 5 mg or 100 mg ascorbate/d/animal over a 90 weeks period, a clear affect on catalase activity in the high-dose ascorbate group as compared to that in the low-dose ascorbate group was not observed.

Key Words ascorbate, catalase, cytotoxicity, guinea pig

Ascorbate is generally known to defend tissues against damage by free radicals because of its antioxidant properties (1–3). On the other hand, ascorbate has the paradoxical ability to act as a pro-oxidant in the presence of metal ions (4–7). Although there have been many reports on the inhibitory effect of high concentrations of ascorbate on cell growth in vitro (8–11) because of H₂O₂ found in aqueous solutions of ascorbate (12–14), the general opinion is that intake of high amounts of ascorbate are not toxic in vivo, and rather contribute to good health. It is generally considered that metal ions inducing the auto-oxidation of ascorbate are sequestered in vivo and that excess ascorbate is excreted in the urine. In addition,
the presence of large amounts of catalase or some other enzyme which would decompose active oxygen species in tissues may be a mechanism to deter cytotoxicity by high concentrations of ascorbate. Since there are many substances which generate active oxygen species as by-products in the usual metabolic process, the loss of enzymes to decompose active oxygen species would influence homeostasis.

One of our previous papers (15) showed that increasing concentrations of ascorbate in cultured medium resulted in increases of \( \text{H}_2\text{O}_2 \), which induced cytotoxicity, and that the addition of catalase into the medium was useful for protecting cells from cytotoxicity by ascorbate. We have also reported (16) that the ascorbate concentration for the inhibition of cell growth was correlated positively to intracellular catalase activity. From these results, we believe that even though high amounts of \( \text{H}_2\text{O}_2 \) are generated from high concentrations of ascorbate, the presence of catalase with increased activity would be useful for the prevention of cytotoxicity by ascorbate both in vivo and in vitro. On the other hand, the lack of catalase might be a trigger for toxicity by ascorbate.

Many recommend the intake of large amounts of ascorbate because of evidence from both in vivo (17–23) and in vitro (24, 25) studies. However, there has been little investigation of the physiological effects of high concentrations of ascorbate using cultured cells. This may be because ascorbate at high concentrations would damage the cells so that they could not be examined. If the addition of catalase in medium could prevent cytotoxicity by ascorbate, then the addition of catalase would result in a new experimental method for the study of cultured cells and high concentrations of ascorbate. In other words, the necessity of adding catalase in the medium would indicate that the difference in experimental results between in vitro and in vivo studies may be caused by a lack of catalase in vitro.

Based on this hypothesis, this study examined the protective effect of catalase against cytotoxicity by ascorbate using the 3T6 cultured cell line. Since catalase seems to play an important role in the physiological effects of ascorbate, we investigated the effect of ascorbate on catalase activity in various tissues of guinea pigs. The catalase activity of guinea pigs whose diets were supplemented with high dosages of ascorbate was compared with that of animals receiving little or no such supplementation.

**MATERIALS AND METHODS**

*Cell culture.* The 3T6 cell line (fibroblasts derived from mouse embryo), obtained from American Type Culture Collection (Rockville, MD, USA), was cultured in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical, Tokyo, Japan). The medium was supplemented with 5% fetal bovine serum (DMEM-5), penicillin (Meiji Seika, Tokyo, Japan), kanamycin sulfate (Meiji Seika), NaHCO3 (Otsuka, Tokyo, Japan) and L-glutamine (Wako Pure Chemical Industries, Osaka, Japan). To investigate the effect of catalase on cell growth, the final concentrations of 0.5 mM L-ascorbic acid sodium salt (Kanto Chemical, Tokyo, Japan) and 25,
100, 400 and 750 units/mL catalase (Wako Pure Chemical Industries, EC 1.11.1.6) were freshly prepared immediately before use. Cells (1×10⁴/3 mL medium) were placed in 60-mm dishes and incubated at 37°C in a 5% CO₂ environment for 5 d. Medium was changed every two days. For cell counts, using a hemocytometer daily, after removal of the medium, the cell layer was rinsed twice with phosphate-buffered saline (PBS(−), Nissui Pharmaceutical) and dispersed by EDTA and trypsin (GIBCO, NY, USA). To investigate whether cytotoxicity by ascorbate at final concentrations of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15 and 20 mM can be prevented by the presence of 750–5,000 units/mL catalase, cells (1×10⁴/3 mL medium) were incubated for 4 d without medium change. The morphology of the cells was observed by microscope daily (15).

Animals. Male Hartley guinea pigs at the age of 3 weeks were obtained from Clea Japan (Tokyo, Japan) and housed in individual cages in a temperature-controlled (25 ± 2°C) room with 55 ± 5% humidity and a 12-h light-dark cycle. The care of these animals conformed to the Guidelines for Animal Experimentation of Ochanomizu University. Guinea pigs were given free access to tap water. Six guinea pigs for each treatment were anesthetized by intraperitoneal injection of sodium pentobarbital after which brain, heart, lung, liver, spleen, stomach, small intestine, kidneys, adrenals and muscle tissue were obtained.

Short-term experiment. After animals were provided with food (containing 0.5 mg ascorbic acid/30 g food/d) for 7 d, ascorbic acid dissolved in 5% sucrose water (100 mg/mL/animal) was administered orally. The tissues, excised after fasting for 6 h, were used for the measurement of catalase activity.

Long-term experiment. Guinea pigs at the age of 3 weeks were kept for 90 weeks. Three types of commercial diets (CG-7*, Clea Japan), whose concentrations of ascorbic acid as analyzed by HPLC were 4.24 ± 0.67 mg, 202 ± 70 mg and 1,700 ± 111 mg/kg diet (mean ± SE), were blended at appropriate ratios in consideration of food intake during feeding periods and body weight of animals. Animals were divided into low-dose and high-dose groups which received 5 mg or 100 mg ascorbic acid/kg body wt/d, respectively. After 0 (untreated), 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 and 90 weeks of feeding, six guinea pigs per treatment per feeding period were sacrificed to obtain the tissues as described above. The tissues were stored at −30°C until analysis of catalase activity, which was performed within 2 weeks.

Measurement of ascorbic acid. Ascorbic acid in the feed was extracted with 2% (w/v) metaphosphoric acid solution. After centrifugation of the extract, ascorbic acid in the supernatant was analyzed by HPLC-ECD (VMD-101A, Yanaco, Tokyo, Japan). The mobile phase consisted of 30 mM potassium dihydrogenphosphate and 0.1 mM EDTA-2Na (adjusted to pH 2.3). Separation was achieved with an ODSII column (GL Science, Japan), and flow rate was 0.6

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*Composition of the commercial diet except vitamin C was as follows (g/kg diet): water, 88; crude protein, 183; crude fat, 39; fiber, 130; ash, 103; energy, 1,135 kJ/kg diet.
mL/min. The applied potential of ECD was 800 mV.

**Measurement of catalase activity.** In order to investigate the effect of ascorbic acid on catalase activity whether in cultured cells or tissues, 1,600 units/mL of catalase was incubated in each medium (DMEM-0) with 0, 0.1, 1 or 10 mM ascorbic acid for 40 h at 37°C. Catalase activity in the medium was measured after 2, 18, 24 and 40 h incubation. Tissues from guinea pigs were homogenized in phosphate buffer (30 mM, pH 7.0). After centrifugation of the homogenate, catalase activity of the supernatant was determined by the spectrophotometric disappearance of H$_2$O$_2$ (26). Decomposition of 10 mM H$_2$O$_2$ was measured for 0–60 s at UV 240 nm.

**RESULTS**

Figure 1 shows the growth curve of 3T6 cells cultured in the absence or presence of 0.5 mM ascorbate and 25–750 units/mL catalase. Although cells supplemented with 0.5 mM ascorbate did not grow, the addition of catalase promoted cell growth. The cell number with ascorbate in the presence of catalase was slightly higher than that without ascorbate, though the difference was not significant. Differences in the degree of catalase activity did not affect cell growth.

Table 1 shows the ascorbate concentrations that did not produce cytotoxic morphology such as the shrinking of cells, which could be observed as described in the photograph in a previous report (15). When catalase of 5,000 units/mL was added to the medium, cytotoxic morphology was not observed in cells cultured for 1 d even at concentrations of 15 mM ascorbate. However, after 3 d culturing, cytotoxic morphology was observed at more than 3 mM ascorbate. The concentra-

![Fig. 1. Growth curve of 3T6 in the presence of catalase. Cells were cultured in DMEM-5 in the absence (○) or presence (▲, □, △, ○, ■) of 0.5 mM ascorbate. Catalase was added in medium at 0 (▲), 25 (□), 100 (△), 400 (○) and 750 (■) units/mL. SDs were less than the size of the symbols.](https://example.com/fig1.png)
Table 1. The concentration of ascorbate at which cytotoxicity occurs with catalase.

| Ascorbate (mM) | Catalase (units/mL) | 5,000 | 4,000 | 3,000 | 2,000 | 1,500 | 1,000 | 750 |
|---------------|---------------------|-------|-------|-------|-------|-------|-------|-----|
|               | Day 1 2 3 4        | Day 1 2 3 4 | Day 1 2 3 4 | Day 1 2 3 4 | Day 1 2 3 4 | Day 1 2 3 4 | Day 1 2 3 4 | Day 1 2 3 4 |
| 20            | + + + +            | + + + + | + + + + | + + + + | + + + + | + + + + | + + + + | + + + + |
| 15            | − + + +            | − + + + | + + + + | + + + + | + + + + | − + + + | + + + + | + + + + |
| 10            | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 9             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 8             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 7             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 6             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 5             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 4             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 3             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 2             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |
| 1             | − + + +            | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + | − + + + |

+: “Cytotoxicity” was detected at this concentration of ascorbate.
−: “Cytotoxicity” was not detected at this concentration of ascorbate.

The appearance of cytotoxic morphology, such as shrinking of cells which could be observed as described in the photograph of a previous report (15), was determined as “cytotoxicity.”
tion of ascorbate at which cytotoxicity occurred with 5,000 units/mL catalase was slightly higher than that with 750 units/mL catalase throughout the culture period. It became lower as a function of culture day, and finally cytotoxicity by ascorbate at a concentration of more than 2 mM was not prevented by catalase of any activity. These results indicate that catalase does not provide protection against cytotoxicity induced by high concentrations of ascorbate.

Figure 2 shows the effect of ascorbate on catalase activity regardless of the presence of cultured cells. Catalase activity in the medium did not change for 40 h in the absence of ascorbate. In the presence of ascorbate, catalase activity was reduced as a function of incubation time. The decline in catalase activity with 10 mM ascorbate was steeper than that with 0.1 mM ascorbate; after 40 h no catalase activity was detected with 10 mM ascorbate. The higher the concentration of ascorbate, the greater the decrease in catalase activity.

The effect of ascorbate on catalase activity in living tissues was expressed as the ratio of catalase activity in various tissues of guinea pigs after 6 h of administration of ascorbate as compared to that of non-administered animals (short-term experiment, Table 2). The administration of ascorbate resulted in a decrease in the average catalase activity in all tissues examined except the liver and adrenals. The catalase activity of brain, lungs and muscle in administered animals was approximately 78% of that in non-administered animals.

Figure 3 shows changes in catalase activity in the various tissues of guinea pigs subjected to long-term administration of ascorbate (90 weeks), which was compared between two dosage groups. In terms of age-related change of catalase
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Table 2. Change in catalase activity in various tissues of guinea pigs by the administration of ascorbate.

| Tissue      | Catalase activity in non-administered animals (units/mg tissue)* | Catalase activity in ascorbate-administered animals (µg/g tissue)* | Ratio of catalase activity (%)** |
|-------------|---------------------------------------------------------------|-----------------------------------------------------------------|----------------------------------|
| Brain       | 0.301±0.09                                                   | 0.233±0.03                                                     | 77.4                             |
| Heart       | 1.51±0.09                                                    | 1.49±0.12                                                      | 98.7                             |
| Lungs       | 11.0±1.3                                                     | 8.53±0.48                                                      | 77.8***                          |
| Liver       | 425±43                                                       | 473±35                                                         | 111                              |
| Spleen      | 10.2±0.48                                                    | 9.90±1.1                                                       | 97.0                             |
| Stomach     | 7.55±0.45                                                    | 5.99±0.49                                                      | 79.3***                          |
| Small intestine | 3.63±0.26                                               | 3.20±0.26                                                      | 88.2                             |
| Kidneys     | 12.6±1.3                                                     | 12.0±1.5                                                       | 95.1                             |
| Adrenals    | 35.2±1.7                                                     | 43.8±4.9                                                       | 124                              |
| Muscle      | 0.546±0.03                                                   | 0.426±0.05                                                     | 78.0                             |

* Values are means±SE of 6 animals.
** Ratio of catalase activity (%) = catalase activity in tissues of ascorbate-administered animals / catalase activity in tissue of non-administered animals.
*** Significantly different p < 0.05.

activity, all tissues examined here had the peak point of catalase activity during weeks 18 to 33 and then the activity decreased. In animals less than 33 weeks of age, catalase activity in the high-dose group tended to be higher than that in the low-dose group. After 33 weeks of age, a high dose of ascorbate had little affect on catalase activity in the brain, heart, lung, liver, spleen, stomach, small intestine, adrenal or muscle. On the other hand, in animals more than 53 weeks of age, catalase activity in the kidney of the high-dose group tended to be lower than that of the low-dose group.

DISCUSSION

Megadoses of ascorbate have been recommended because some in vivo investigations to evaluate the effects of high concentrations of ascorbate have shown clinical significance other than the prevention of scurvy (17–25). However, ascorbate at concentrations ranging between 0.05 to 4 mM have been shown to suppress cultured cell growth (6–8, 10, 27–30). This inhibitory effect has been ascribed to H₂O₂ derived from ascorbate (12–15). The question remains as to whether toxicity by ascorbate can occur in vivo. Cytotoxicity by ascorbate was induced easily in cultured cells with a low catalase activity for decomposition of H₂O₂ (16). However, since living tissues have large amounts of catalase (31), they are not expected to be affected by ascorbate even though H₂O₂ would be generated from high concentrations of ascorbate. From the above hypothesis, it was further
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Fig. 3. Changes in catalase activity in the tissues of guinea pigs as a function of age. Values are means ± SE of 6 animals in each age. Significantly different from the values of 5 mg/d group (p < 0.05).
hypothesized that any concentration of ascorbate would fail to inhibit cell growth if catalase was supplied to cells in cultures at high levels of activity.

Contrary to our expectations, the cytotoxicity induced in 3T6 cells by ascorbate at concentrations of more than 2 mM was not prevented even though as much as 5,000 units/mL catalase was added to the medium (Table 1). The decrease of catalase activity in the medium in the presence of various concentrations of ascorbate (Fig. 2) may explain why cytotoxicity by high concentrations of ascorbate was not prevented even in the presence of catalase with higher activity in the medium. That is, the increased degradation of catalase activity by higher concentrations of ascorbate indicates that ascorbate is a catalase inhibitor in a cultured system. Therefore, large amounts of \( \text{H}_2\text{O}_2 \), as a toxic agent derived from high concentrations of ascorbate, were not decomposed because of the inactivation of catalase by ascorbate. Li et al. (32) reported that the exposure of SOD to \( \text{H}_2\text{O}_2 \) or ascorbate-Fe(III) resulted in a significant loss of its activity. Further, the inactivation of lactate dehydrogenase by ascorbate via oxygen free radicals has been reported (33, 34). Considering that ascorbate can act as a pro-oxidant and a wide variety of proteins and enzymes were modified by metal catalyzed oxidation (35), the decrease of catalase activity by ascorbate observed in this study may be due to damage to the catalase by oxygen free radicals derived from ascorbate.

In order to investigate whether the degradation of catalase activity by ascorbate in vitro is also induced in vivo, catalase activity in various tissues of the guinea pig supplemented with ascorbate for short- and long-term periods was measured. That the catalase activity in tissues, except for the liver and adrenals, 6 h after the administration of 100 mg ascorbate was lower than in non-administered animals (Table 2) would support the results in vitro that ascorbate reduced catalase activity in the medium. However, catalase activity in the brain, heart, lung, liver, spleen, stomach, small intestine, adrenal and muscle did not differ between groups fed 5 mg or 100 mg ascorbate from 3 to 93 weeks of age (Fig. 3). These results of short- and long-term administration of ascorbate suggest that almost all tissues possess the ability to recover over time, even though ascorbate degrades catalase activity transiently.

On the other hand, lower catalase activity was found in the kidneys of the high-dose ascorbate group after 53 weeks of age. This may support the results in vitro and in short-term experiments that high concentrations of ascorbate induce the degradation of catalase activity. As the results observed in the short-term experiments that, in tissues such as the liver and adrenals, catalase activity was not degraded by ascorbate, the effect of ascorbate on catalase activity may differ among tissues. Thus, the inhibition of catalase activity by ascorbate is not believed to be universal. Further, it seems that this property is not always evident because decreasing catalase activity in the kidneys was observed after 53 weeks of age.

Young et al. (36) stated that the use of high-dose ascorbate supplementation as an antioxidant intervention in patients with diabetes should be approached with caution until there is a clearer understanding of these actions. Other recent reports
(37,38) indicate possible detriments along with the benefits of ascorbate. This study indicates that ascorbate could inhibit catalase activity both in vitro and in vivo. Considering that enzymes which decompose active oxygen species such as catalase and superoxide dismutase are among the functional physiological systems that protect the body from H₂O₂ or other active oxygen species which damage organs, it is suspected that under certain conditions such as aging and sickness, and with certain tissues, a decrease in catalase activity may trigger oxidative damage in vivo as well as cytotoxicity in cultured cells. From this point, continuous long-term dosages of high levels of ascorbate may require the caution as described by the above reports. However, in vivo, this transient inhibition of catalase activity by ascorbate seems to be reversed to achieve homeostasis. Therefore, under normal conditions, cytotoxicity by ascorbate as observed in cultured cells would not appear in vivo. Though massive concentrations of ascorbate have beneficial effects on health definitively, further studies on their simultaneous effects are required from various sides in terms of physiological functions.

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