*Free Radical Research*, February 2005; 39(2): 213–220



# Dual role of vitamin C in an oxygen-sensitive system: Discrepancy between DNA damage and dell death

MINYI SHI[1], BAOHUI XU[1], KAZUKO AZAKAMI[1], TOMOHIRO MORIKAWA[1], KUNITOMO WATANABE[2], KANEHISA MORIMOTO[3], MASAHARU KOMATSU[1], KOHJI AOYAMA[1], & TORU TAKEUCHI[1]

[1]*Department of Environmental Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan,* [2]*Division of Anaerobe Research, Life Science Research Center, Gifu University, Gifu, Japan, and* [3]*Department of Social and Environmental Medicine, Course of Social Medicine, Osaka University Graduate School of Medicine, Suita, Japan*

Accepted by Professor N. Taniguchi

*(Received 15 October 2004; In revised form 4 November 2004)*



**Abstract**
Although vitamin C is considered to act both as pro-oxidant and antioxidant, the mechanisms underlying these actions are still unclear. Using the oxygen-sensitive system of a strict anaerobe, *Prevotella melaninogenica*, we investigated both the pro-oxidant and antioxidant mechanisms of vitamin C. In the presence of vitamin C, the 8-hydroxydeoxyguanosine (8OHdG) formation induced by oxygen exposure was enhanced, probably due to the action of vitamin C on hydrogen peroxide generated during oxygen exposure: while catalase almost completely suppressed the enhancing effect of vitamin C, 8OHdG formation induced by hydrogen peroxide was enhanced by vitamin C. By contrast, the presence of vitamin C inhibited bacterial cell death, membrane damage, and lipid peroxidation induced by oxygen exposure. Sodium azide showed similar effects to vitamin C, thus the antioxidant action of vitamin C may be due to its quenching of the singlet oxygen generated in this system. Both the pro-oxidant and antioxidant effects of vitamin C were observed only in acidic conditions.

**Keywords:** *Vitamin C, oxidative DNA damage, lipid peroxidation, bacterial cell killing, membrane damage, Prevotella melaninogenica*

**Abbreviations:** *8OHdG, 8-hydroxydeoxyguanosine; dG, deoxyguanosine; DPBS, Dulbecco's phosphate buffered saline; ESR, electron spin resonance; FDA, fluorescein diacetate;* $H_2O_2$*, hydrogen peroxide; HPLC, high-performance liquid chromatography; MDA, malondialdehyde;* $NaN_3$*, sodium azide; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substance; TEP, 1,1,3,3-tetraethoxypropane*

## Introduction

Numerous exogenous agents and endogenous processes are capable of inducing free radicals *in vivo* [1]. In particular, reactive oxygen species (ROS) are produced continuously during normal cellular metabolism, exposure to radiation, or metabolic activation by certain chemicals [2]. These endogenously generated ROS are held by some to cause such damage as to be a major contributing factor in aging and numerous degenerative processes including cancer, heart disease, cataracts, and cognitive dysfunction [3–6]. Two defense mechanisms that limit the levels of ROS and thus prevent oxidative damage,

Correspondence: T. Takeuchi, Department of Environmental Medicine, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan. Tel.: +81-99-275-5288. Fax: +81-99-265-8434. E-mail: takeuchi@m.kufm.kagoshima-u.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400022129

have evolved in organisms. One is mediated by antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [2,7], the other by dietary antioxidant chemicals contained in fruits and vegetables [8,9].

Of the small-molecule antioxidants, vitamin C has received the most attention. Experimental and epidemiological studies have reported that by scavenging physiologically relevant ROS and reactive nitrogen species, vitamin C has anticarcinogenic and chemopreventive actions [10,11]. Moreover, vitamin C is an effective water-soluble antioxidant that, in plasma, can prevent lipid peroxidation induced by peroxyl radicals or the gas-phase of cigarette smoke, and possibly protect against cardiovascular disease [12,13]. On the other hand, substantial evidence has shown that vitamin C may also act as an pro-oxidant, depending upon the environment in which it is present [14]. For example, it can induce apoptotic cell death in human myelogenous leukemic cell lines[15] and shows genotoxic effects in some test systems [16,17]. More recently, the pro-oxidative effect of vitamin C on biomacromolecules has been receiving increasingly great attention but much remains to be understood. Further research is essential to elucidate the specific local effects of vitamin C in biological systems [18,19].

In a previous study, using a strict anaerobe, we established a highly oxygen-sensitive biological system [20]. Now we have used this system to investigate the role of vitamin C. In particular, we investigated the effect of vitamin C on the generation of 8-hydroxydeoxyguanosine (8OHdG), typically present when there is oxidative DNA damage, [21–23] and on lipid peroxidation, membrane damage, oxidative protein damage, and the killing of bacterial cells exposed to oxygen.



## Materials and methods

### *Materials*

L-Ascorbic acid (vitamin C) was obtained from Katayama Chemicals, Inc. (Osaka, Japan). Superoxide dismutase (SOD) was purchased from Wako Pure Chemicals, Inc. (Osaka, Japan). Sodium azide ($NaN_3$), sodium dodecyl sulfate (SDS) and HPLC grade acetonitrile were supplied by Nacalai Tesque, Inc. (Kyoto, Japan). Polyoxyethylenesorbitan monolaurate (Tween 20) and β-carotene came from Sigma-Aldrich Chemie Gmbh. (Steinheim, Germany) and catalase from Boehringer-Mannheim (Mannheim, Germany). Hydrogen peroxide was supplied by Santoku Chemicals, Inc. (Tokyo, Japan), while fluorescein diacetate (FDA), thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (TEP), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). S7150 Oxyblot™ Protein Oxidation Detection Kits were obtained from Chemicon International (Serologicals Corporation, GA).

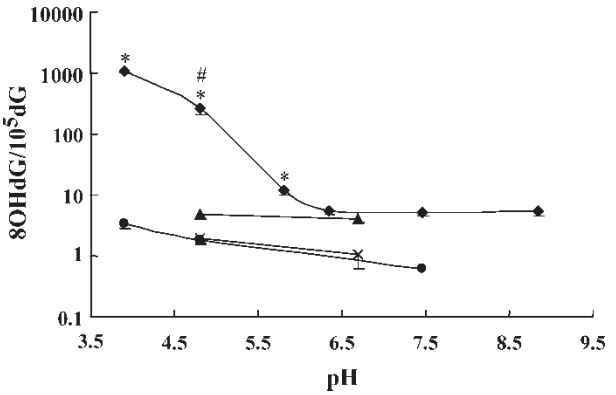


Figure 1. Correlation of pH with the effects of vitamin C on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen under different pH conditions in the presence or absence of 10 mM vitamin C and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. ×, without oxygen exposure and without vitamin C; ▲, oxygen exposure without vitamin C; ●, without oxygen exposure but with vitamin C; ◆, oxygen exposure with vitamin C. Data are expressed as mean − SE. $^{\star}P < 0.05$, indicates significantly enhanced presence of 8OHdG, compared with samples at pH 7.46. $^{\#}P < 0.05$, indicates significantly enhanced presence of 8OHdG, compared with ▲ samples (at the same pH exposed to oxygen without vitamin C).

### *Growth and collection of bacteria*

*Prevotella melaninogenica* (*P. melaninogenica*) (GAI5490, strict anaerobe) cultures were grown on Brucella HK agar (Kyokuto Seiyaku Kogyo, Tokyo, Japan) in an anaerobic incubator (model 1024, Forma Scientific Inc., Marietta, OH) at 37°C as previously described [20]. After incubation for 3 days, bacteria were harvested and suspended in Dulbecco's phosphate buffered saline (DPBS, pH 7.0, Nikken Seibutsu, Kyoto, Japan, used in Figure 1 and Table I experiments) or citrate-buffered saline (CBS, pH 5.0, used in

Table I. Correlation of pH and effect of vitamin C on bacterial cell survival

| pH | $O_2-$ | $O_2+$ | vC($O_2-$) | vC($O_2+$) |
|---|---|---|---|---|
| 7.0 | 100 | 0 | 108.4 ± 29.2 | 0 |
| 5.0 | 100 | 0.0036 ± 0.0016 | 83.7 ± 3.4 | 0.1040 ± 0.029* |

Bacterial cells suspended in neutral (pH 7.0) or acidic (pH 5.0) DPBS buffer were exposed or left unexposed to oxygen and then incubated at 37°C for 3 h in the presence or absence of 10 mM vitamin C. Bacterial cell survival was determined as described in "Material and Methods" section. Samples without oxygen exposure and without vitamin C ($O_2-$) served as control (100%). The cell survival of other samples was calculated as percent of control. $O_2-$, without oxygen exposure without vitamin C; $O_2+$ oxygen exposure without vitamin C; vC($O_2-$), without oxygen exposure with vitamin C; vC($O_2+$), oxygen exposure with vitamin C. Results from three separate experiments are presented as mean ± SE. $^{\star}P < 0.05$ compared with $O_2+$ sample.

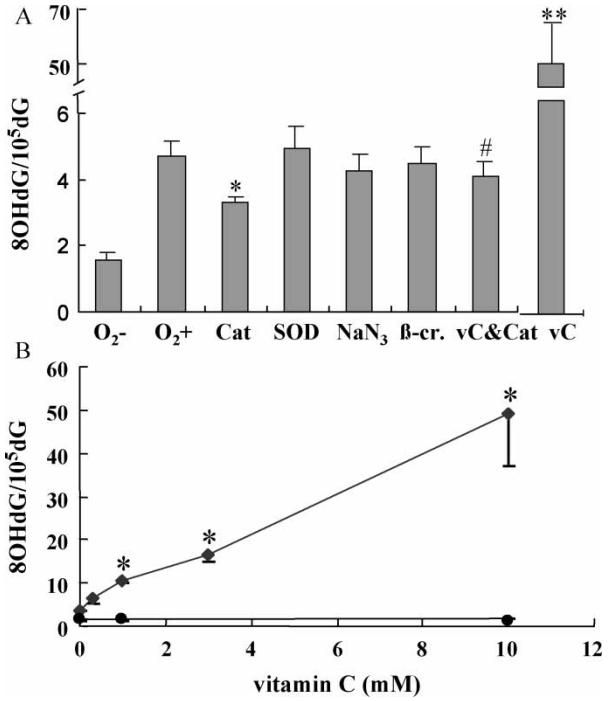


Figure 2. Effects of ROS scavengers (A) and dose-dependent effect of vitamin C (B) on induction of 8OHdG by oxygen. Bacterial cells were exposed to oxygen in the presence of vitamin C and/or ROS scavengers at pH 5.0 and then incubated at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. (A) $O_2-$, without oxygen exposure and without vitamin C or scavengers; $O_2+$, oxygen exposure but without vitamin C or scavengers; the following samples were exposed to oxygen in the presence of various reagents. Cat, catalase (1000 units/ml); SOD, superoxide dismutase (300 units/ml); $NaN_3$, sodium azide (1 mM); β-cr., β-carotene (10 μM); vC and Cat, co-addition of vitamin C (10 mM) and catalase (1000 units/ml); vC, vitamin C (10 mM). $^{\star}P < 0.05$, compared with samples exposed to oxygen ($O_2+$), indicates significant suppression of, and $^{\star\star}P < 0.05$ significant increase in, induction of 8OHdG; $^{\#}P < 0.05$, compared with samples with vC, indicates significantly suppressed induction of 8OHdG. (B) ●, without oxygen exposure but with vitamin C; ◆, oxygen exposure with vitamin C. $^{\star}P < 0.05$ compared with samples exposed to oxygen without vitamin C. Results from three independent experiments are presented as mean ± SE.

Figures 2–5 and Table II experiments) under oxygen-free conditions. Bacterial cell density was spectrophotometrically measured with 660 nm light and adjusted to density of 2.6 during all experiments.

### *Exposure of bacteria to oxygen and hydrogen peroxide*

Bacteria were exposed to oxygen ($O_2$) by bubbling gaseous $O_2$ at 100 ml/min for 30 s through 1 ml samples of bacterial cell suspensions in 15 ml centrifuge tubes. Then tubes were tightly sealed and samples were incubated at 37°C for 1 or 3 h.

Exposure to hydrogen peroxide ($H_2O_2$) was carried out by incubating similar bacterial cell suspensions with various concentrations of $H_2O_2$ at 37°C for 3 h.

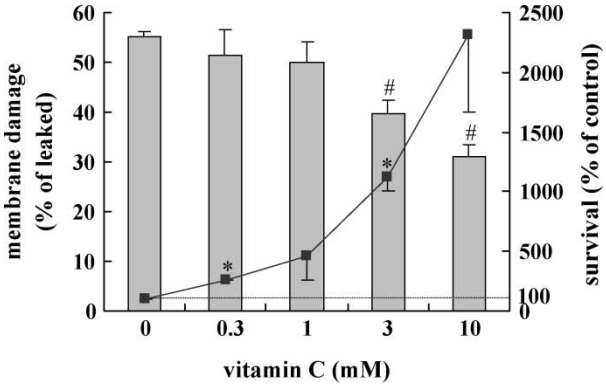


Figure 3. Effects of vitamin C on bacterial membrane damage (bar graph) and killing of bacterial cells (line graph) associated with oxygen exposure at pH 5.0. Membrane damage and bacterial cell mortality were investigated and analyzed as described in "Materials and Methods" section. Results from three independent experiments are presented as mean ± SE, except for mortality at 10 mM, for which two independent experiments were performed. $^{\star}P < 0.05$ compared to the survival of samples exposed to oxygen without vitamin C. $^{\#}P < 0.01$, compared to the membrane damage of samples exposed to oxygen without vitamin C.

All enzymes or other reagents were added to suspensions of *P. melaninogenica* just before exposure to $O_2$.

### *Evaluation of bacterial cell killing*

After being exposed to $O_2$ and incubated for 3 h, bacterial cell suspensions were appropriately diluted with DPBS and spread on Brucella HK agar plates. Colonies were counted after 3–4 days incubation under anaerobic conditions at 37°C. Survival was calculated by dividing the number of colonies growing per dish from test samples by the number of colonies

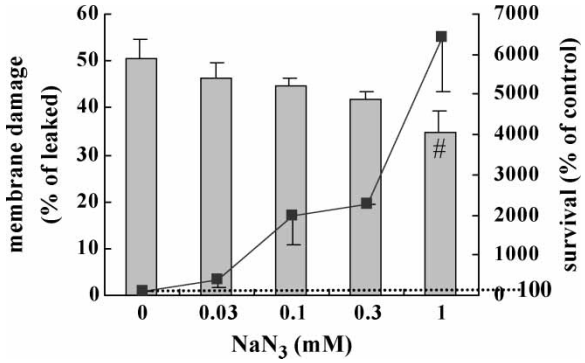


Figure 4. Effects of sodium azide on bacterial membrane damage (bar graph) and bacterial cell mortality (line graph) associated with oxygen exposure at pH 5.0 Bacterial cell mortality and membrane damage were investigated and analyzed as described in "Materials and Methods" section. For membrane damage, results from three independent experiments are presented as mean + SE and, for mortality, from two independent experiments (mean − range). $^{\#}P < 0.05$, compared to samples exposed to oxygen in the absence of sodium azide, indicates significantly decreased membrane damage.

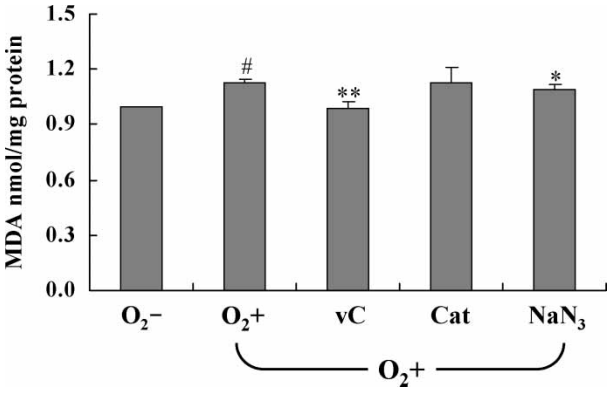


Figure 5. Effects of vitamin C and ROS scavengers on oxygen-induced lipid peroxidation. Bacteria were exposed to oxygen in the presence of vitamin C or ROS scavengers at pH 5.0 and subsequently incubated at 37°C for 1 h. Results are shown as mean + SE from four independent experiments. See Fig. 2A for concentrations of vC (vitamin C) and scavengers and explanation of $O_2-$ and $O_2+$. $^{\#}P < 0.05$, compared with $O_2-$ samples, indicates significantly greater induction of lipid peroxidation. $^{\star}P < 0.05$ and $^{\star\star}P < 0.01$,compared with $O_2+$ samples, indicate significantly suppressed lipid peroxidation.

growing per dish from control samples (without $O_2$ exposure and without presence of vitamin C or other agents, such as enzymes).

*Detection of 8OHdG*

The DNA of the bacteria was extracted and digested under anaerobic conditions [24]. Quantities of 8OHdG and deoxyguanosine (dG) were determined by HPLC with electrochemical detection and UV absorption as previously described [25]. Oxidative DNA damage (8OHdG levels) was expressed as the molar ratio of 8OHdG per $10^5$ dG.

*Determination of membrane damage*

Fluorescein diacetate (FDA) assay has been employed to assess cell membrane integrity [26,27]. FDA permeates the cells and is hydrolyzed by cellular esterases to form fluorescein, which is fluorescent and remains trapped within the cell [28]. Fluorescent cells are assumed, therefore, to have intact cell membranes. Bacterial membrane damage was evaluated by using flow cytometry to measure the leakage of fluorescent dye (FDA) from labelled bacteria.

Bacterial cells were incubated with FDA (4.8 μM) at 37°C for 15 min under oxygen-free conditions to allow the permeation of FDA, then these labelled cells were exposed to $O_2$ as described above. Exposed bacterial samples were incubated at 37°C for 1 h, then diluted 1000 times with DPBS and evaluated using flow cytometry and CellQuest software (FACScan, Becton Dickinson, Franklin Lakes, NJ). Membrane damage was expressed as percentage of leaked fluorescence compared with fluorescence of unexposed cells.

*Detection of lipid peroxidation*

Malondialdehyde (MDA) was used as an index of lipid peroxidation and measured using a previously described TBA method [29,30]. After oxygen exposure, bacterial samples (1 ml) were incubated at 37°C for 1 h, after which 100 μl 0.69% KCl and 100 μl 4.86% SDS were added. Then the mixture was centrifuged at 12,000 rpm. The supernatant was incubated at 95°C for 1 h in TBA working solution (0.2% TBA solution in 0.1 M sodium acetate buffer, pH 2.5). After cooling to room temperature, the samples were centrifuged at 10,000*g* for 10 min. Supernatant was used for the HPLC analysis. The TBA–MDA adduct was separated on an octadecylsilane column (TSK gel ODS 80Ts, TOSOH Corporation, Tokyo, Japan) and monitored with a fluorescence detector at 515 nm excitation and 553 nm emission. TEP was used as a standard. Lipid peroxidation was calculated as MDA equivalents and expressed as nmol MDA per milligram protein measured by the Bradford assay.

*Statistical analysis*

Results are presented as mean ± SEM (standard error of the mean). Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Table II. Effect of vitamin C on induction of 8OHdG by hydrogen peroxide.

| | Vitamin C (mM) | | | |
|---|---|---|---|---|
| $H_2O_2$ (mM) | 0.0 | 0.3 | 1.0 | 3.0 |
| 0.2 | 18.2 ± 0.3 | 32.2 ± 1.4⋆ | 61.2 ± 5.1⋆ | 97.5 ± 11.2⋆ |
| 0.5 | 25.5 ± 1.4 | 46.3 ± 1.0⋆ | 85.2 ± 0.6⋆ | 156.5 ± 18.3⋆ |
| 1.0 | 34.5 ± 2.5 | NT | 103.0 ± 14.3⋆ | 182.8 ± 23.7⋆ |

With the indicated concentrations of $H_2O_2$, bacterial cells were incubated at pH 5.0, in the absence or presence of vitamin C, at 37°C for 3 h. 8OHdG levels were determined as described in "Materials and Methods" section. Data from three separate experiments are presented as mean ± SE. NT, not tested. $^{\star}P < 0.05$ compared with samples which incubated with the same concentration of hydrogen peroxide in the absence of vitamin C.

## Results

*Effects of vitamin C on oxygen-induced bacterial cell killing and the relationship of pH to oxidative DNA damage*

After bacterial cells were exposed to oxygen in neutral or acidic conditions, oxidative DNA damage and bacterial cell killing were evaluated. Oxygen significantly induced DNA damage under both neutral and acidic conditions. The resultant amount of 8OHdG

did not significantly vary depending on pH (Figure 1). When vitamin C was added to bacterial cells, however, the oxidative DNA damage due to oxygen exposure significantly increased, but only in samples with acidic conditions (pH $<$ 6; $P < 0.05$). Table I shows the effect of vitamin C on bacterial cell survival in different pH conditions. If bacteria were not exposed to $O_2$, vitamin C made no difference to survival in either neutral or acidic conditions. Both in the presence and absence of vitamin C, at pH 7.0, oxygen exposure killed all bacteria. On the other hand, at pH 5.0, vitamin C suppressed the cell killing due to $O_2$ exposure.

*Effects of scavengers on oxygen-induced 8OHdG*

Exposure to $O_2$ resulted in 8OHdG formation in bacterial cells. We examined the effects of ROS scavengers on oxygen-induced 8OHdG at pH 5.0. As shown in Figure 2A, superoxide scavenger SOD, and singlet oxygen scavengers sodium azide and β-carotene did not suppress the formation of 8OHdG in the presence of oxygen. By contrast, catalase significantly inhibited oxygen-induced 8OHdG formation. As described above, vitamin C on its own did not increase 8OHdG, however, it did dose-dependently enhance the production of 8OHdG in the presence of oxygen (Figure 2B). Catalase almost completely inhibited this effect (Figure 2A).

*Effect of vitamin C on $H_2O_2$-induced 8OHdG*

Table II shows the effect of vitamin C on oxidative DNA damage induced by $H_2O_2$: 8OHdG increased depending on the amount of $H_2O_2$ present, and vitamin C also dose-dependently enhanced this production of 8OHdG in the presence of $H_2O_2$.

*Effects of vitamin C on oxygen-induced bacterial cell killing and membrane damage*

Incubation of vitamin C with bacterial samples while they were exposed to oxygen dose-dependently increased survival of bacterial cells (Figure 3). At lower concentrations, vitamin C provided less effective protection, but at 0.3 and 3 mM, the number of surviving cells significantly increased compared to bacteria exposed to $O_2$ without vitamin C.

Vitamin C dose-dependently suppressed membrane damage induced by oxygen exposure (Figure 3). As with the effect of vitamin C on cell survival, significant protection was observed at 3 and 10 mM. In this experimental system using *P. melaninogenica*, no membrane damage was induced by $H_2O_2$.

*Effects of ROS scavengers on oxygen-induced cell killing*

Vitamin C suppressed oxygen-induced bacterial cell killing (Figure 3). To gain insight into the mechanism of this suppressive effect, we examined the effects of ROS scavengers on oxygen-induced bacterial cell killing at pH 5.0. In line with results obtained in our previous report [20], catalase suppressed the cell killing. Bacterial colony numbers per milliliter of bacterial suspension ($n = 3$–5 per category) were $6500 \pm 3000$ when vitamin C alone was added, $4300 \pm 1600$ with catalase alone, and $57{,}400 \pm 12{,}600$ in the presence of both vitamin C and catalase. Compared with vitamin C or catalase alone, the co-addition of catalase and vitamin C significantly suppressed the cell killing ($P < 0.05$). In a way similar to the effects of vitamin C, sodium azide, a typical singlet oxygen scavenger, concentration-dependently suppressed both oxygen-induced cell killing and membrane damage (Figure 4). Another typical singlet oxygen scavenger β-carotene had similar effects to vitamin C and sodium azide. Superoxide scavenger SOD, however, had no effect (data not shown).

*Effects of scavengers and vitamin C on lipid peroxidation induced by oxygen*

Testing for lipid peroxidation, we found increased MDA (evaluated as a TBA-reactive substance, TBARS) after exposure to oxygen (Figure 5). While both vitamin C and sodium azide significantly suppressed the production of MDA, no suppression was found with catalase or SOD (data not shown).

## Discussion

Although vitamin C is one of the most important nutrients and antioxidants in the human body, pro-oxidative effects have also been reported. To elucidate the working of this nutrient, we investigated some of the actions of vitamin C in a simple biological system. We have already established a highly oxygen-sensitive biological system using a strict anaerobe [20], which has proved useful in investigating the biological effects of ROS and the activities of antioxidants and pro-oxidants. In the current study, we investigated both the pro-oxidative and antioxidative effects of vitamin C on the system. When there was exposure to $O_2$, the presence of vitamin C enhanced oxidative DNA damage even while suppressing bacterial cell killing, membrane damage, and lipid peroxidation. These actions are likely to result from different mechanisms and such findings further demonstrate the usefulness of this bacterial system for investigating the effects of ROS and antioxidants.

We have already reported that, after *P. melaninogenica* is exposed to $O_2$, hydrogen peroxide and superoxide are generated and 8OHdG is induced [31]. The suppressive effect of catalase on 8OHdG induction indicates that hydrogen peroxide induces oxidative DNA damage in the system. When vitamin C was added to the system, by itself it had no effect on 8OHdG induction, however, under acidic conditions, when there was exposure to $O_2$, the presence of vitamin C did significantly enhance oxygen-induced 8OHdG formation. Meanwhile, catalase almost completely suppressed this vitamin C enhancement of 8OHdG formation. Such findings indicate that this pro-oxidative action of vitamin C is most likely due to its capacity for enhancing the toxicity of hydrogen peroxide in the system. Using the same system, under the same pH conditions as during oxygen exposure, vitamin C enhanced $H_2O_2$-induced 8OHdG formation (Table II). Although membrane permeable hydrogen peroxide did induce oxidative DNA damage (Table II), it is unlikely to have a direct effect on DNA [32]. Rather, its effect is most probably mediated by the generation of a hydroxyl radical by the Fenton reaction, which involves iron-dependent reduction of hydrogen peroxide [33–35]. Vitamin C is a cellular reducing agent that is likely to be able to replace superoxide in reducing $Fe^{3+}$ to $Fe^{2+}$ and, consequently, promote the formation of hydroxyl radicals when hydrogen peroxide is present [14,36–38]. Several studies have reported the genotoxicity of vitamin C and the interaction with iron was suggested to be the likely mechanism [39,40] Moreover, in the Fenton reaction, free iron readily acts as a catalyst. Studies with *E. coli* and yeast have shown that free iron exists in growing cells [41,42]. In our system, when we added phenanthroline to *P. melaninogenica*, the bacterial cells turned red, indicating the presence of free iron. Spectrophotometric analysis confirmed that 5 μM of phenanthlorine chelatable iron was present in the cell suspension. Since iron is more soluble in acidic conditions, it becomes more available for redox reactions [43,44]. Our findings indicate that vitamin C enhances oxygen-induced 8OHdG formation only in acidic conditions. Thus, when local conditions are acidic, we speculate that vitamin C acts as a pro-oxidant by reducing free iron, which accelerates the Fenton reaction.

Serious DNA damage is considered to induce cell death, including necrosis and apoptosis [2,45,46]. In the present study, however, with vitamin C present in acidic conditions, an inverse relation was observed between 8OHdG levels and cell survival. This suggests that vitamin C enhances the survival of cells with DNA damage, and this may help to explain the reported carcinogenicity of vitamin C [47,48].

Vitamin C suppressed the oxygen-induced death of bacterial cells. It also suppressed membrane damage, which is a likely factor in cell death, and suppressed lipid peroxidation, which may be a cause of membrane damage. We found that while catalase also reduced cell mortality, it did not suppress membrane damage (data not shown) or lipid peroxidation. Meanwhile, at 1.0 mM, although $H_2O_2$ killed the bacteria completely, it did not induce membrane damage. These findings suggest that something other than $H_2O_2$ itself induces cell death. Several reports have described the important role of singlet oxygen in killing bacterial cells [49–52]. At the same time, singlet oxygen-induced cell death has been associated with the reactivity of singlet oxygen with lipids and/or proteins [53–55]. Consequently, we investigated the effects of singlet oxygen scavengers on our system. Both sodium azide and β-carotene suppressed cell death. Because sodium azide is so easy to handle, we further investigated its effects on membrane damage and lipid peroxidation and found that the presence of sodium azide had effects similar to the presence of vitamin C. The inhibiting effect of sodium azide on lipid peroxidation and membrane damage indicates that singlet oxygen induces lipid peroxidation and bacterial cell membrane damage. Without oxygen exposure, sodium azide in concentrations up to 1 mM did not affect the survival of *P. melaninogenica*. It seems reasonable to conclude that the cell death that we observed in this study was at least partly due to the presence of singlet oxygen, which altered lipids and damaged the membrane. Reports have shown that, after incubating vitamin C with chemically generated singlet oxygen, vitamin C can scavenge singlet oxygen [56,57]. Although the generation of singlet oxygen has been reported in some kinds of bacterium [58], we are not able to find evidence of its generation in our system. Although we were unable to detect the presence of singlet oxygen, and in spite of the efforts of others with ESR[59,60] and HPLC with fluorescent detection [61], we still find the most plausible explanation for the antioxidative effect of vitamin C is its action on singlet oxygen. Compared with the effect of vitamin C or catalase on their own, the co-presence of catalase and vitamin C significantly and greatly suppressed cell killing, and this also supports such a conclusion. When we studied protein oxidation, however, we found no evidence, evaluated by the subsequent amounts of protein carbonyls present, that exposure to oxygen induced protein oxidation (data not shown).

The increased damage caused by vitamin C to the DNA of bacterial cells suggests that vitamin C was present in the bacterial cells. Indicating that the transport of vitamin C into bacterial cells is possible, the presence of L-ascorbate permease has been reported for *E. coli* and has been identified in association with a wide variety of bacteria [62]. Vitamin C is a diacid with $pK1 = 4.2$ and $pK2 = 11.6$. Studies on the stability of vitamin C show that the rate of vitamin C oxidation increases as pH rises [63]. *In vitro*, vitamin C concentrations remained

stable in acidic, but fell significantly during 24 h in neutral (pH 7.2), gastric aspirate [64]. The stability of vitamin C in acidic condition may explain why, in our system, both the pro-oxidant and antioxidant effects of vitamin C were observed only in acidic conditions.

In conclusion, in our *P. melaninogenica* model, oxygen exposure induced significant oxidative stress. In particular, there is evidence that the presence of oxygen resulted in the formation of $H_2O_2$ and singlet oxygen, which, in turn, induced oxidative DNA damage and cell death. Vitamin C showed both pro-oxidative activity by enhancing the oxidative DNA damage and, antioxidative activity by decreasing cell death, membrane damage, and lipid peroxidation subsequent to oxygen exposure. The dual role of vitamin C is probably due to its contrary action on two kinds of oxidative stress. In our model, the pro-oxidative effect of vitamin C probably results from its enhancement of hydrogen peroxide toxicity. At the same time, the antioxidative effect is likely due to its quenching of singlet oxygen. Numerous pathologies have been associated both with oxidative stress and with low tissue-pH values [65,66]. Therefore, before recommending the use of vitamin C as a dietary antioxidant supplement, it would be prudent to consider the local physiological environments in which, after consumption, vitamin will be present.



## Acknowledgements

This work was supported by Grand-in-Aid for Scientific Research awarded by the Ministry of Education, Science, Sports and Culture of Japan (#14370140). The authors express thanks to Drs Yasuteru Urano and Tetsuo Nagano (Graduate School of Pharmaccutical Sciences, The University of Tokyo) for providing a fluorescent probe for singlet oxygen, and to Mr. David Eunice for copyediting the manuscript.

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