Protective effect of vitamin C on 8-hydroxy-2′-deoxyguanosine level in peripheral blood lymphocytes of chronic hemodialysis patients

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Background. This study focused on the effect of vitamin C on the 8-hydroxy-2′-deoxyguanosine (8-OHdG) level of cellular DNA, as well as 8-oxoguanine-DNA glycosylase 1 (hOGG1) and human MutT homologue (hMTH1) gene expression in peripheral blood lymphocytes of chronic hemodialysis patients.

Methods. Sixty chronic hemodialysis patients (35 men and 25 women) were recruited to participate in a randomized, placebo-controlled study. Treatment order is block-randomized with intravenous sodium ascorbate (vitamin C, 300 mg) or placebo (0.9% saline), administered postdialysis three times a week. We evaluated 8-OHdG level, intracellular reactive oxygen species (ROS) production, and gene expression of hOGG1 and hMTH1 in peripheral blood lymphocytes by using high-performance liquid chromatography (HPLC) electrochemical detection method, flow cytometric analysis, and reverse transcription-polymerase chain reaction (RT-PCR), respectively.

Results. A total of 51 patients completed the study (26 in placebo group and 25 in vitamin C group). Mean 8-OHdG levels significantly decreased in total subjects following 8 weeks of vitamin C supplementation (22.9 vs. 18.8/10⁶ dG, P < 0.01). The decrease in 8-OHdG levels after vitamin C supplementation was also noted in the patients with ferritin <500 or ≥500 μg/L and transferrin saturation (TSAT) <50 or ≥50% (P < 0.05). But 8-OHdG levels had no significant changes in total patients or in the four subgroups of patients treated with placebo as compared to their baselines. Intracellular ROS production by lymphocytes from the four subgroups of patients, either spontaneous (P < 0.05) or phorbol-12-myristate-13-acetate (PMA)-stimulated (P < 0.001), was significantly reduced after 8 weeks vitamin C supplementation. Steady-state hOGG1 mRNA levels were significantly up-regulated at 24 hours after vitamin C administration (P < 0.05), but hMTH1 mRNA levels were not. The changes in the spontaneous and PMA-stimulated ROS production, and an up-regulation of hOGG1 mRNA expression were not observed in patients treated with placebo as compared to their baselines.

Conclusion. Vitamin C supplementation in chronic hemodialysis patients can reduce the lymphocyte 8-OHdG levels and intracellular ROS production, as well as up-regulate hOGG1 gene expression for repair. There is no compelling evidence for an in vivo pro-oxidant effect of vitamin C on lymphocyte DNA base oxidation, even in the status of increased iron stores.

Cumulative data have shown that oxidative damage to biomolecules such as DNA, lipids, and proteins has been implicated as a causal factor for a number of complications in patients undergoing chronic hemodialysis [1–3]. Cellular DNA is particularly susceptible to oxidative injury in chronic hemodialysis patients because there is an imbalance between reactive oxygen species (ROS) and antioxidant defenses [4, 5], or repair enzymes [6]. Of the more than 20 different oxidative DNA base lesions, 8-hydroxy-2′-deoxyguanosine (8-OHdG) appears to be the most abundant and mutagenic [7, 8]. 8-OHdG has emerged as a novel marker for the assessment of oxidative DNA damage in ROS-mediated diseases [9, 10]. Our recent works have further demonstrated that 8-OHdG contents in cellular DNA provide a reliable measure of steady-state oxidative DNA damage in peripheral blood leukocytes for chronic hemodialysis patients [4, 5]. Increased leukocyte 8-OHdG levels are elicited by the abundant ROS elaborated from leukocytes with metabolic activation after blood-membrane contact during hemodialysis [5, 11]. The augmented DNA oxidation is further aggravated by the impaired oxidant scavenging system of plasma as substantiated by decreased plasma levels of vitamins C and E and glutathione in chronic hemodialysis patients [4–6].

Vitamin C (ascorbate) is depleted in the fluids in vivo under conditions of oxidative stress such as smoking [12] and inflammation associated with rheumatoid arthritis.
Hemodialysis has also been shown to decrease antioxidant defenses, which results in an increased requirement of chronic hemodialysis patients for the supplementation of vitamin C, the main hydrophilic antioxidant of the body [14, 15]. Recently, therapeutic approaches based on parenteral administration of vitamin C have received increasing attention in most hemodialysis units to promote better anemia control and iron utilization. The beneficial effect on recombinant erythropoietin response can be observed not only for chronic hemodialysis patients with normal iron status [16], but also for those having a high ferritin level of >500 to 800 μg/L [17–19]. Theoretically, vitamin C scavenges ROS and reactive nitrogen species and may thereby prevent oxidative damage to important biologic macromolecules. Investigators reported that an increase in lipid peroxidation is prevented by administration of high doses of vitamin C (504 mg per session) during hemodialysis using a traditional dialyzer of cellulose membrane [15]. However, to our best knowledge, there is no study to examine whether vitamin C exhibits a protective role in DNA oxidation for chronic hemodialysis patients. In contrast, vitamin C can act as a pro-oxidant by reduction of redox active transition metal ions (i.e., iron and copper) through Fenton chemistry. The availability of catalytic transition metals in human blood may be the key factor in determining vitamin C-related oxidative paradox [13, 20]. Since vitamin C administration facilitates iron mobilization in chronic hemodialysis patients [16–19], it potentially might be hazardous, especially in those with iron overload. In such patients, vitamin C might promote electron exchange and enhance iron toxicity to cellular constituents. Therefore, the aims of the present study is to elucidate whether vitamin C acts as an antioxidant in vivo to attenuate oxidative DNA damage in lymphocytes of chronic hemodialysis patients, or paradoxically as a pro-oxidant to amplify DNA oxidation.

Repair of DNA that contains oxidized bases or other abnormal bases is essential for maintaining the accuracy of genetic information. In mammalian cells, 8-OHdG causes misreading through G to T and A to C transitions, which are reported to be responsible for its mutagenicity [8–10]. Therefore, cells recognize 8-OHdG as a threatening lesion that requires rapid removal. Repair of DNA containing oxidized bases involves complex pathways at both the blood and cellular levels. Multiple repair mechanisms that remove 8-OHdG from DNA [21, 22], or prevent its incorporation into DNA [23, 24], have evolved. 8-Oxoguanine-DNA glycosylase 1 (hOGG1) and human MutT homologue (hMTH1) are two representative enzymes for repair of its respective nucleotide 8-hydroxyguanine (8-OHG) [21–24]. In healthy subjects receiving vitamin C supplementation, Cooke et al [25] observed a significant increase in the levels of serum and urine 8-OHdG, measured using competitive enzyme-linked immunosorbent assay (ELISA) method. This finding has been inferred as being due to stimulation of DNA repair enzymes by vitamin C. Therefore, to determine in vivo effects of vitamin C on the major lesion of DNA base oxidation and regulation of gene expression for its repair enzymes, 8-OHdG contents in cellular DNA and intracellular ROS production, as well as mRNA expression of hOGG1 and hMTH1 were measured in peripheral blood lymphocytes of chronic hemodialysis patients before and after supplementation of vitamin C. In addition, the changes in these parameters were analyzed in our patients having a serum ferritin level of >500 μg/L or transferrin saturation (TSAT) of >50%.

**METHODS**

**Patients and study protocol**

To assess whether or not vitamin C supplementation has an in vivo antioxidant effect on cellular DNA of peripheral blood lymphocytes, a total of 60 patients (35 men and 25 women) undergoing chronic hemodialysis at the two dialysis centers of our affiliated hospital were recruited to participate in a prospective, randomized, placebo-controlled study. Exclusion criteria included age <20 years; duration on hemodialysis protocols for <3 months before the study; habit of tobacco smoking; disorders such as diabetes mellitus, malignancy, chronic inflammatory diseases and acute infections; consumption of vitamin C or E; and treatment with oral or intravenous iron supplements, angiotensin-converting enzyme (ACE) inhibitors or anti-inflammatory drugs 3 months prior to enrollment. The causes for chronic renal failure were glomerulonephritis (N = 24), interstitial nephritis (N = 11), nephrosclerosis (N = 11), polycystic kidney disease (N = 6), and shrunken kidney with unknown etiology (N = 8). Their mean age was 58 ± 12 years and average duration on hemodialysis was 46 ± 25 months. Dialytic procedure was a standard bicarbonate session, performed with single-use dialyzers equipped with cellulose membrane (Terumo, Tokyo, Japan) of 1.75 m² effective surface area. Hemodialysis was performed three times weekly for 12 to 13.5 hours/week. Dialysis machines were sterilized daily, and water treatment circuits and tanks were sterilized weekly. The colony count of microorganisms in water used to prepare dialysis fluid did not exceed 200 colonies/mL. Endotoxin levels in dialysate, detected weekly by amebocyte lysate test (LAL) (Chromogenix, Charleston, SC, USA), were less than 0.01 EU/mL.

The randomized, placebo-controlled study was carried out for 8 weeks. In the 8-week period, chronic hemodialysis patients were randomly assigned to receive supplementation with vitamin C or normal saline (placebo). Treatment order is block-randomized with the use of computerized-generated random numbers. Ten milliliters...
of sodium ascorbate (total vitamin C, 300 mg) or 10 mL of 0.9% saline was administered intravenously for 5 minutes postdialysis three times weekly for 8 weeks. Blood samples were taken on two occasions, before supplementation started and after 8 weeks supplementation. All patients fasted for 12 hours immediately before the blood samples were taken. Any ongoing medications were continued without changes in dosage during the study. The patients were also required to complete a 3-day food diary, which was used to estimate the daily intake of vitamin C before the investigation and at the study completion [26]. The protocol was approved by the Committee on Human Research at Taipei Veterans General Hospital. Informed consent was obtained from each of the study subjects.

Labatory measurements

In each study subject, whole blood was collected by venipuncture predialysis and divided into aliquots for the subsequent analyses to be performed. For transferrin bound iron levels and iron-binding capacity blood serum samples were separated. An aliquot of 1 mL plasma was mixed with 1 mL of 100 g/L metaphosphoric acid (MPA) and stored at −70°C until measurement of ascorbate concentration. In addition, 2 mL of blood was withdrawn into a heparinized vacutainer tube for flow cytometric analysis of intracellular ROS production by lymphocytes, and 10 mL of blood was withdrawn into an ethylenediaminetetraacetic acid (EDTA)-containing vacutainer for isolation of lymphocytes. Lymphocytes were separated from 10 mL of whole blood on Ficoll-Hypaque density gradients, washed twice in phosphate-buffered saline (PBS) (pH 7.4), and centrifuged again. The cells recovered were confirmed to be 95% lymphocytes and frozen at −70°C until use for determination of 8-OHdG content in the cellular DNA.

Iron in serum was determined using the commercial kit by an autoanalyzer (Hitachi 736–60) (Naka, Japan). Total iron-binding capacity was measured by the TIBC Microtest (Daichi, Tokyo, Japan) and serum ferritin by radioimmunoassay (Incstar, Stillwater, MN, USA). Transferrin saturation (TSAT) was calculated as the serum iron concentration/TIBC × 100. Serum C-reactive protein (CRP) was measured by an immunoturbidimetric assay using high-performance liquid chromatography (HPLC) equipped with an EC detector (Bioanalytical Systems, West Lafayette, IN, USA) as described previously [4–6, 29]. Deoxynucleosine (dG) (Sigma Chemical Co., St. Louis, MO, USA) and 8-OHdG were used as standards. The amount of 8-OHdG was measured by the method using high-performance liquid chromatography (HPLC) equipped with an EC detector (Bioanalytical Systems, West Lafayette, IN, USA) as described previously [4–6, 29]. Deoxynucleosine (dG) (Sigma Chemical Co., St. Louis, MO, USA) and 8-OHdG were used as standards. The 8-OHdG level is expressed as the number of 8-OHdG molecules per 10⁶ dG. Intraassay coefficients of variance (CV) ranged from 4% to 8% and interassay CV ranged from 5% to 10%, where the lower numbers refer to the CV for the high standard and the higher numbers refer to the CV for the low standard.

Flow cytometric analysis of intracellular ROS production

Two 100 μL aliquots of each sample were analyzed for ROS production, one at baseline and the other after activation with phorbol-12-myristate-13-acetate (PMA) (Sigma Chemical Co., St. Louis, MO, USA). Leukocytes were harvested from blood samples after lysis of red blood cells by a lysis solution (0.15 mol/L NaHCO₃, 10 mmol/L NaHCO₃, and 10 mM EDTA, pH 7.4). After centrifugation for 10 minutes at 350 × g and 4°C, leukocyte pellet was suspended in 1.5 mL of Hanks’ balanced salt solution (pH 7.2). Cell viability was determined by trypan blue exclusion. Leukocytes were then incubated at 37°C for 5 minutes with 1.5 μL of 20 mmol/L

Measurement of 8-OHdG content

Total DNA of lymphocytes was extracted by the pronase/ethanol method [28] with some modifications. Briefly, nuclear fractions were obtained by centrifugation at 1000 × g for 10 minutes after gentle homogenization of peripheral blood lymphocytes in 10 mL of 5 mmol/L Tris-HCl buffer (pH 7.6) containing 1% Triton X-100, 320 mmol/L sucrose and 10 mmol/L MgCl₂. The nuclear fraction was resuspended vigorously in 700 μL of standard sodium citrate (SSC) (5 mmol/L sodium citrate and 20 mmol/L sodium chloride, pH 6.5). After adding 200 μL of pronase E (20 mg/mL in SSC), 800 μL of Sarkosyl (1.5% in 20 mmol/L EDTA, and 20 mmol/L Tris-HCl, pH 8.5) and 100 μL of 5% butylated hydroxytoluene (BHT) in methanol, the mixture was incubated for 6 hours at 45°C. After incubation, following the addition of 800 μL of TE buffer (10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH7.5) and 200 μL of 7.5 mol/L ammonium acetate, cooled ethanol (−20°C) was carefully added up to 70% while mixing. Precipitated DNA was stored overnight in 95% ethanol containing 0.01% BHT. The amount of 8-OHdG was measured by the method using high-performance liquid chromatography (HPLC) equipped with an EC detector (Bioanalytical Systems, West Lafayette, IN, USA) as described previously [4–6, 29]. Deoxynucleosine (dG) (Sigma Chemical Co., St. Louis, MO, USA) and 8-OHdG were used as standards. The 8-OHdG level is expressed as the number of 8-OHdG molecules per 10⁶ dG. Intraassay coefficients of variance (CV) ranged from 4% to 8% and interassay CV ranged from 5% to 10%, where the lower numbers refer to the CV for the high standard and the higher numbers refer to the CV for the low standard.
2',7'-dichlorofluorescin diacetate (DCF-DA) (Molecular Probes, Eugene, OR, USA). After labeling, leukocytes were incubated for 30 min at 37°C in the presence or absence of 100 ng/mL of PMA. Total leukocytes were subjected to flow cytometry analysis (FACSort®) (Becton-Dickinson, San Jose, CA, USA) for measurement of intracellular production of ROS ($O_2^·−$ and $H_2O_2$) [30]. The lymphocyte population was determined by gating on a forward scatter and side scatter dot plot as described previously [11]. Intracellular ROS production was expressed as mean fluorescence of 2',7'-dichlorofluorescein (DCF), a product liberated from DCFH, which is hydrolyzed to nonfluorescent polar derivative from DCF-DA by intracellular esterases, and is highly fluorescent after oxidation by $H_2O_2$. ROS production was then monitored every 10 minutes on FACSort by measuring the intensity of fluorescence emitted at 525 nm for DCF. Data for each sample were calculated by the CellQuest® software (Becton-Dickinson) on a power Macintosh 6100/66 computer.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from lymphocyte ($5 \times 10^6$) using a RNA-zol kit (Cinna/Biotex Laboratories International Inc., Friendwood, TX, USA). First-strand cDNA was synthesized by Ready-to-GoTM RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using Moloney murine leukemia virus-derived reverse transcriptase according to the procedure recommended by the manufacturer. Complementary DNA was amplified in 100 μL total volume containing 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.0), 10 mmol/L deoxynucleoside triphosphate (dTTP), 1.5 mmol/L MgCl₂, 1 U Taq polymerase, and 10 pmol of specific PCR primers. The sets of primers used for PCR amplification were 5'-ACTGTGGTCATGAGTCC TTC-3' (sense) and 5'-AGGGGAA CAGT AGCTGTC-3' (antisense) for hOGG1 [31], yielding a 203 bp PCR product; and 5'-CTCAGCG AGTTCTCCTGG-3' (sense) and 5'-GGAGTGGAAAC CAGT AGCTGTC-3' (antisense) for hMTH1 [23], yielding a 334 bp PCR product. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was coamplified with primers of 5'-GGGCGTCACGGATTTGGTC-3' (sense) and 5'-ACTGTGGTCATGAGTCC TTC-3' (antisense), yielding a 516 bp PCR product as the internal standard. The thermoprofile consisted of 30 to 38 cycles of denaturation at 94°C for 15 seconds, annealing at 56°C for 15 seconds, and extension at 72°C for 30 seconds, preceded by an initial denaturation step at 94°C for 2 minutes and followed by a terminal extension of 5 minutes at 72°C. The number of cycles used allowed quantification without saturation [32]. Amplification products were separated by electrophoresis on 2% agarose gel, followed by ethidium bromide staining, and then photographed. The amplification bands were quantified from the film negative by scanning densitometry. Semi-quantitation was done by serial dilution of the input cDNA to measure the mRNA. The proportion of specific gene product to GAPDH product was used for semiquantitative analysis.

Statistical analysis

Statistical analysis was performed using the computer software Statistical Package of Social Science, version 8.0 (1997; SPSS Inc., Chicago, IL, USA). Data are expressed as mean values ± SD. Values for serum ferritin were not normally distributed and reported as means with ranges. Mann-Whitney rank sum test was used for comparison of data from two groups and Pearson's chi-square test for frequency measures. Data for more than two groups were analyzed using Kruskal-Wallis test, followed by multiple comparison tests for significance of differences. Wilcoxon signed ranks test was used for comparison of baselines and data after treatment with vitamin C or placebo. The relationships between 8-OHdG contents in lymphocyte DNA and the potentially explanatory continuous variables were analyzed by Pearson's correlation. A P value of less than 0.05 is considered statistically significant.

RESULTS

Nine patients (five in the placebo group and four in the vitamin C treatment group) had withdrawn by the end of study because of cerebral infarct ($N = 1$), pneumonia ($N = 2$), arteriovenous access thrombosis ($N = 3$), and transfer to peritoneal dialysis ($N = 1$), and renal transplantation ($N = 2$). Finally, a total of 51 patients (26 in placebo group and 25 in vitamin C group) completed the study. In placebo group ($N = 26$), there were 14 patients with ferritin of $< 500 \mu g/L$ and 12 with ferritin of $\geq 500 \mu g/L$, or 14 with TSAT of $< 50%$ and 12 with TSAT of $\geq 50%$. In vitamin C group ($N = 25$), there were 13 patients with ferritin of $< 500 \mu g/L$ and 12 with ferritin of $\geq 500 \mu g/L$, or 15 with TSAT of $< 50%$ and 10 with TSAT of $\geq 50%$ (Table 1). Patients treated with placebo or vitamin C did not differ significantly from each other in terms of age, gender distribution, duration of hemodialysis, hemoglobin values, weekly dose of recombinant erythropoietin, serum CRP, and causes of chronic renal failure in the subgroups stratified by a cutoff value of serum ferritin of 500 μg/L or TSAT of 50%. Mean dietary vitamin C intake of the subjects, as assessed by the food diary, was optimal throughout the study and no significant variation was observed within the study period. Mean daily intake of vitamin C was $129 \pm 58$ mg/day in total subjects and no significant difference was observed between patients receiving placebo and vitamin.
C supplement in the subgroups of patients ($P > 0.05$) (Table 1).

**Plasma ascorbate and serum iron indices**

Overall, supplementation with vitamin C for 8 weeks significantly increased plasma ascorbate concentration from $43.7 \pm 18.6 \mu M/L$ to $88.4 \pm 28.5 \mu M/L$ ($P < 0.001$) (Table 2). Moreover, the increase was significant irrespective of the presupplemental serum ferritin and TSAT ($42 \pm 12 \mu M/L$ to $87 \pm 24 \mu M/L$ in those with ferritin of $<500 \mu G/L$ ($P < 0.005$) and $45 \pm 19.7 \mu M/L$ to $90 \pm 33 \mu M/L$ in those with ferritin of $\geq 500 \mu G/L$ ($P < 0.005$); $42 \pm 14 \mu M/L$ to $87 \pm 31 \mu M/L$ in those with TSAT of $<50\%$ ($P < 0.005$) and $46 \pm 25 \mu M/L$ to $91 \pm 39 \mu M/L$ in those with TSAT of $\geq 50\%$ ($P < 0.01$). In contrast, there were no significant changes in plasma ascorbate concentration after treatment with placebo. As compared with the presupplemental values, no significant differences were observed in serum ferritin and iron concentrations and TSAT after 8 weeks supplementation either with vitamin C or with placebo. A trend to increase TSAT and to decrease serum ferritin levels was only noted in the patients with ferritin of $\geq 500 \mu G/L$ after 8 weeks of vitamin C supplementation, but it did not reach statistical significance ($P > 0.05$) (Table 2).

**Vitamin C reduced intracellular ROS production of lymphocytes**

To evaluate the activated oxygen metabolism of lymphocytes, the intracellular production of ROS in lymphocytes was analyzed by flow cytometry before and after 8 weeks supplementation either with vitamin C or with placebo. The DCF fluorescence intensity was determined for 30 minutes because the fluorescence increased almost linearly up to 30 minutes. Within 30 minutes, the spontaneous ROS production increased above the initial level in unstimulated lymphocytes from total subjects significantly decreased after 8 weeks of supplementary vitamin C ($35 \pm 33\%$ to $7 \pm 15\%$, $P < 0.05$) (Fig. 1). Significant decreases in the spontaneous ROS production of unstimulated lymphocytes were also observed in patients with ferritin of $<500$ or $\geq 500 \mu G/L$ and with TSAT of $<50\%$.  

### Table 1. Characteristics of hemodialysis patients recruited in the study and of patients in the subgroups stratified by cutoff values of serum ferritin of 500 μg/L and transferrin saturation (TSAT) of 50%

<table>
<thead>
<tr>
<th>Cause of renal failure</th>
<th>Patients with serum ferritin &lt;500 μg/L</th>
<th>Patients with serum ferritin $\geq$500 μg/L</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Vitamin C supplement</td>
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<tr>
<td>Glomerulonephritis percentage (%)</td>
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<td>6 (42.9)</td>
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<tr>
<td>Interstitial nephritis percentage (%)</td>
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<td>Nephrosclerosis percentage (%)</td>
<td>9 (17.6)</td>
<td>1 (7.1)</td>
</tr>
<tr>
<td>Polycystic kidneys percentage (%)</td>
<td>6 (11.8)</td>
<td>2 (14.3)</td>
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<td>Unknown cause percentage (%)</td>
<td>6 (11.8)</td>
<td>2 (14.3)</td>
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</table>

<table>
<thead>
<tr>
<th>Cause of renal failure</th>
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<th>Patients with TSAT $\geq$50%</th>
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<td>Vitamin C supplement</td>
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<tr>
<td>Glomerulonephritis percentage (%)</td>
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<td>5 (33.3)</td>
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<tr>
<td>Interstitial nephritis percentage (%)</td>
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<tr>
<td>Unknown cause percentage (%)</td>
<td>3 (21.4)</td>
<td>2 (13.5)</td>
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</tbody>
</table>

*aMann-Whitney U test. bPearson’s chi-square test.*
or ≥50% after vitamin C supplementation \( (P < 0.05) \). But there was no significant difference after treatment with placebo as compared to the baseline values. Likewise, the PMA-stimulated production of ROS by lymphocytes from chronic hemodialysis patients significantly decreased after 8 weeks of supplementary vitamin C \((124 \pm 44\% to 61 \pm 21\%, P < 0.001)\) (Fig. 2). The decrease in PMA-stimulated ROS production by lymphocytes after vitamin C supplementation was also observed in patients with ferritin of <500 µg/L and with TSAT of <50% or ≥50% \( (P < 0.05) \). No significant changes were noted in the PMA-stimulated ROS production by lymphocytes after 8 weeks treatment with placebo.

**Vitamin C attenuated oxidative DNA damage of lymphocytes**

Mean 8-OHdG contents in cellular DNA of lymphocytes significantly decreased following 8 weeks supplementary vitamin C in total 25 subjects \((22.9 \pm 8.7 to 18.8 \pm 8.3/10^6 \text{ dG}, P < 0.01)\) (Fig. 3). The decrease was also noted in the four subgroups of patients after supplementation with vitamin C \((17.2 \pm 6.0 to 14.6 \pm 7.1/10^6 \text{ dG})\) in those with ferritin of <500 µg/L, \( P < 0.05 \) and 29.1 \pm 6.6 to 23.3 \pm 7.3/10^6 \text{ dG} \) in those with ferritin of ≥500 µg/L, \( P < 0.05 \); 21.6 ± 9.4 to 17.2 ± 8.8/10^6 \text{ dG} \) in those with TSAT of <50%, \( P < 0.05 \) and 23.8 ± 8.3 to 17.8 ± 8.1/10^6 \text{ dG} \) in those with TSAT of ≥50%, \( P < 0.05 \). But there were no significant changes in mean 8-OHdG levels in total 26 patients and in the four subgroups of patients receiving placebo treatment as compared to their baseline values. Lymphocyte 8-OHdG at baselines was significantly higher in vitamin C treated patients with ferritin ≥500 µg/L as compared to those with ferritin of <500 µg/L \( (P < 0.05) \), but showed no significant differences in placebo group with ferritin of <500 and ≥500 µg/L or following 8 weeks of placebo and vitamin C treatment \( (P > 0.05) \) (Fig. 3). Univariate analysis disclosed that the decrease in lymphocyte 8-OHdG levels after 8 weeks of supplementary vitamin C significantly correlated with the increase in plasma ascorbate, and the decrease in spontaneous and PMA-stimulated ROS production by lymphocytes in total 25 subjects and in the subgroups of patients with ferritin of <500 or ≥500 µg/L and with TSAT of <50% or ≥50% (Table 3). But these correlations were not observed in 26 patients receiving placebo treatment.

**Table 2.** Plasma ascorbate, serum ferritin, and iron concentrations and transferrin saturation (TSAT) before and after treatment in hemodialysis patients

<table>
<thead>
<tr>
<th>Time weeks</th>
<th>Placebo</th>
<th>Vitamin C supplement</th>
<th>Placebo</th>
<th>Vitamin C supplement</th>
<th>Placebo</th>
<th>Vitamin C supplement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C ( \mu \text{mol/L} )</td>
<td>0</td>
<td>49.7 ± 35.9</td>
<td>43.7 ± 18.6</td>
<td>54.2 ± 38.1</td>
<td>42.2 ± 12.2</td>
<td>44.5 ± 28.9</td>
</tr>
<tr>
<td>8</td>
<td>52.2 ± 42.0</td>
<td>88.4 ± 28.5</td>
<td>57.7 ± 43.8</td>
<td>87.2 ± 23.8</td>
<td>45.9 ± 34.3</td>
<td>89.7 ± 33.2</td>
</tr>
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<td>Serum ferritin ( \mu \text{g/L} )</td>
<td>0</td>
<td>649 (28–2355)</td>
<td>667 (29–826)</td>
<td>342 (28–496)</td>
<td>277 (29–478)</td>
<td>1008 (551–2355)</td>
</tr>
<tr>
<td>8</td>
<td>662 (28–7899)</td>
<td>574 (68–1753)</td>
<td>295 (28–480)</td>
<td>320 (68–482)</td>
<td>1090 (334–2889)</td>
<td>849 (295–1733)</td>
</tr>
<tr>
<td>Serum iron ( \mu \text{g/dL} )</td>
<td>0</td>
<td>100 ± 41</td>
<td>94 ± 49</td>
<td>100 ± 45</td>
<td>107 ± 60</td>
<td>99 ± 36</td>
</tr>
<tr>
<td>8</td>
<td>98 ± 59</td>
<td>89 ± 52</td>
<td>96 ± 61</td>
<td>90 ± 69</td>
<td>101 ± 58</td>
<td>89 ± 29</td>
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<tr>
<td>TSAT %</td>
<td>0</td>
<td>43 ± 17</td>
<td>38 ± 16</td>
<td>40 ± 16</td>
<td>42 ± 20</td>
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<tr>
<td>8</td>
<td>41 ± 22</td>
<td>36 ± 11</td>
<td>32 ± 11</td>
<td>32 ± 9</td>
<td>50 ± 27</td>
<td>39 ± 12</td>
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</table>

Comparison between baselines and 8 weeks by Wilcoxon signed rank test.
Wilcoxon signed rank test.

The percentages of increased in fluorescence above the resting level within 30 minutes are shown in all chronic hemodialysis patients, patients with ferritin values of <500 and ≥500 µg/L, and patients with transferrin saturation (TSAT) of <50 and ≥50%, respectively. Brackets indicate SD. *P < 0.05 vs. 0 week by Wilcoxon signed rank test.

Vitamin C up-regulated hOGG1 mRNA expression in lymphocytes

To explore the effect of vitamin C on the mRNA levels of DNA repair gene, first we compared the hOGG1 and hMTH1 mRNA levels of lymphocytes among normal healthy subjects (N = 5), uremic patients not receiving dialysis yet (N = 5), and chronic hemodialysis patients (N = 20). Then we assessed the changes in hOGG1 and hMTH1 mRNA expression for 24 hours after vitamin C administration (300 mg) in chronic hemodialysis patients. Lymphocyte 8-OHdG levels were highest in chronic hemodialysis patients, followed by undialyzed patients then by normal healthy controls (25.4 ± 8.5 vs. 12.5 ± 5.3 vs. 6.3 ± 4.7/10⁶ dG, ANOVA P < 0.001). Steady-state hOGG1 mRNA level significantly increased in undialyzed patients, and further rose in chronic hemodialysis patients (P < 0.01), increasing to 2.8-fold above that of controls (Fig. 4). hMTH1 mRNA level of chronic hemodialysis patients significantly increased to 2-fold above that of controls (P < 0.05), but showed no significant difference from that of undialyzed patients (Fig. 4). Vitamin C treatment resulted in a time-dependent rise of hOGG1 mRNA expression, increasing to twofold above the baselines at 24 hours. In contrast, there was no detectable effect on the levels of hMTH1 mRNA for 24 hours (data not shown). Overall, the administration of vitamin C to chronic hemodialysis patients significantly up-regulated hOGG1 mRNA levels at 24 hours in those with ferritin of <500 µg/L (N = 10) and of ≥500 µg/L (N = 10) (P < 0.05) (Fig. 5), but the change in hMTH1 mRNA levels was not observed. There was no effect of placebo treatment on the expression of hOGG1 or hMTH1 mRNA at 24 hours in chronic hemodialysis patients (Fig. 5). Twenty chronic hemodialysis patients were reanalyzed and stratified by TSAT of <50% (N = 14) and of ≥50% (N = 6). An up-regulation of hOGG1 mRNA levels at 24 hours was also observed after the administration of vitamin C to both subgroups of patients.


<table>
<thead>
<tr>
<th>Variable</th>
<th>All Patients $(N = 25)$</th>
<th>Ferritin $&lt; 500 \mu g/L$ $(N = 13)$</th>
<th>Ferritin $\geq 500 \mu g/L$ $(N = 12)$</th>
<th>TSAT $&lt; 50%$ $(N = 15)$</th>
<th>TSAT $\geq 50%$ $(N = 10)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta$Plasma ascorbate</td>
<td>$-0.649^b$</td>
<td>$-0.473^a$</td>
<td>$-0.456^a$</td>
<td>$-0.543^a$</td>
<td>$-0.477^a$</td>
</tr>
<tr>
<td>$\Delta$Spontaneous ROS production &lt;br&gt;(% of baseline within 30 minutes) in lymphocytes</td>
<td>$0.486^a$</td>
<td>$0.558^a$</td>
<td>$0.325$</td>
<td>$0.512^a$</td>
<td>$0.392$</td>
</tr>
<tr>
<td>$\Delta$PMA-induced ROS production &lt;br&gt;(% of baseline within 30 minutes) in lymphocytes</td>
<td>$0.634^b$</td>
<td>$0.567^a$</td>
<td>$0.488^a$</td>
<td>$0.600^a$</td>
<td>$0.516^a$</td>
</tr>
<tr>
<td>$\Delta$Serum ferritin</td>
<td>$0.162$</td>
<td>$0.233$</td>
<td>$0.156$</td>
<td>$0.107$</td>
<td>$0.276$</td>
</tr>
<tr>
<td>$\Delta$Serum iron</td>
<td>$0.212$</td>
<td>$0.148$</td>
<td>$0.271$</td>
<td>$0.284$</td>
<td>$0.105$</td>
</tr>
<tr>
<td>$\Delta$TSAT</td>
<td>$0.321$</td>
<td>$0.281$</td>
<td>$0.357$</td>
<td>$0.145$</td>
<td>$0.122$</td>
</tr>
</tbody>
</table>

Abbreviations are: PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; TSAT, transferrin saturation; $\Delta$, difference between before treatment and 8 weeks after treatment for each variable. Univariate analysis is performed using Pearson correlation. Lymphocyte 8-OHdG and serum ferritin values are not normally distributed and are thus transformed by natural logarithm. Data are expressed as correlation coefficient ($r$).

$^aP < 0.05$.  
$^bP < 0.005$.

(P < 0.05), but the change in hMTH1 mRNA levels was still not noted.

**DISCUSSION**

Oxidatively induced DNA damage in peripheral blood leukocytes is known to be increased in end-stage renal disease (ESRD) patients receiving hemodialysis [4–6]. In this study, we further demonstrated that oxidative DNA damage, as assessed using the novel marker of 8-OHdG in peripheral blood lymphocytes, is increased in both chronic hemodialysis and nondialyzed patients, and more elevated in the former. The level of 8-OHdG measured in lymphocytes is an integration of a number of parameters, including the cellular redox status, antioxidant defense mechanisms, and ROS production. Oxidative stress is increased in chronic hemodialysis patients, and peripheral blood neutrophils and lymphocytes may be an important source for ROS due to metabolic activation after blood-membrane contact during hemodialysis [5, 11]. Protection from oxidant injury involves complex pathways at both the blood and cellular levels. Vitamin C acts as a potent water-soluble antioxidant in biologic fluid by scavenging pathologically relevant ROS and reactive nitrogen species [13, 20]. Our previous data expressing significantly decreased plasma level of ascorbate provide insight into the defective redox status in chronic hemodialysis patients [4–6]. These findings are in line with the investigations of decreased plasma concentration of vitamin C in chronic hemodialysis patients due to increased consumption caused by increased oxidative stress, loss through dialysis process, and inadequate dietary intake [33, 34]. Accordingly, increased oxidative DNA damage in lymphocytes may in part result from low vitamin C and increased hemodialysis-derived oxidants. In this study the most compelling finding shows that vitamin C decreased lymphocyte 8-OHdG contents and markedly reduced intracellular ROS production by lymphocytes, either spontaneous or PMA-stimulated, in both chronic hemodialysis patients with normal and increased iron stores (Figs. 1 to 3). The correlation of decreases in 8-OHdG in DNA with increases in plasma vitamin C concentration following supplementation (Table 3) suggests strongly a protective role of vitamin C against oxidative DNA damage.

The human data on the role of vitamin C in protecting against oxidatively induced DNA damage are controversial and appear inconsistent. Some investigators have reported a reduction in ex vivo or in vivo DNA oxidation in lymphocytes after vitamin C supplementation in healthy volunteers [25, 35, 36], whereas others have shown no change [37] or even yielded mixed results with a decreased in some types of oxidized DNA bases and an increase in others [38, 39]. However, this inconsistency is likely due to technical problems associated with gas chromatography–mass spectrometry analysis in some studies [38, 39]. HPLC-EC detection method used in the present study appears to be gaining favor on account of its minimal ex vivo oxidation artifacts and low basal levels of oxidative DNA damage. The lymphocyte 8-OHdG levels measured in our healthy subjects are much lower than those by other investigators [40]. In addition, rigorous procedures, such as the addition of antioxidants during isolation and hydrolysis of DNA, avoiding the use of phenol, and storing samples under nitrogen, were employed in our study to minimize and control for sources of experimental error [9, 10]. Another crucial point with regard to human supplementation studies is that proper placebo controls often are not included [38, 39]. To avoid a questionable experimental design [20], our study has been conducted with a proper placebo group throughout the entire duration of the study.
Numerous studies showed vitamin C inhibits oxidative DNA damage in isolated or cultured cells exposed to ultraviolet visible light [41]; nevertheless, in vitro studies are often confounded by the pro-oxidant effect of vitamin C in the presence of free transition metals [42]. Since vitamin C administration facilitates iron mobilization in chronic hemodialysis patients [16–19], there are concerns that it might promote electron exchange and enhance iron toxicity to cellular constituents, especially in those patients with increased iron stores. However, the hypothesis is not supported by two recent human studies with vitamin C and iron cosupplementation to healthy subjects [39, 43]. There is no overall increase in total DNA base damage; furthermore, a significant decrease in leukocyte 8-OHdG levels was observed at the end of the two studies [39, 43]. In our study a reduction in lymphocyte 8-OHdG...
levels and intracellular ROS production by vitamin C is also demonstrated in chronic hemodialysis patients both with normal and increased iron stores. Our data corroborate their findings [39, 43] and reveal no compelling evidence for a pro-oxidant effect of vitamin C on DNA base damage in chronic hemodialysis patients, even in those with increased iron stores.

In our study, following 8 weeks vitamin C supplementation, the generation of ROS by lymphocytes is reduced significantly, showing that vitamin C is involved in the inhibition of ROS formation. This response is also observed in glutathione-depleted HL-60 cells [44] and neutrophils of chronic heart failure patients receiving long-term vitamin C therapy [45]. Therefore, it seems unlikely that the antioxidant effect of vitamin C relies only on its extracellular scavenging effect. Vitamin C is actively transported into cells through an insulin-dependent transport system [46]. There is a wide range of intracellular concentrations of vitamin C in human cells and tissues. Levine et al. [47] found that lymphocytes accumulate 3.5 mmol/L ascorbic acid at oral intake of 100 to 200 mg of vitamin C daily, which corresponds to a plateau plasma concentration of 56 to 66 µmol/L. In the present study, plasma concentration of vitamin C is less than 50 µmol/L prior to supplementation, indicating that intracellular vitamin C in lymphocytes of chronic hemodialysis patients is not saturated at baseline. After 8 weeks of supplementation, plasma vitamin C concentration increases to a mean level of 88.4 µmol/L, at which saturates lymphocyte vitamin C levels. Thus, scavenging effects of vitamin C may be mainly intracellular. An inhibition of ROS production points to that vitamin C is primarily responsible for neutralizing ROS to prevent cellular DNA damage. In addition, ROS-induced DNA oxidation is likely due to generated intermediate free radicals, and vitamin C may have its major quenching effect at this level. Paradoxically, Chen et al. [48] reported that, at higher concentrations such as 10^3 and 10^4 µmol/L, vitamin C induces ROS generation determined in vitro by lucigenin-enhanced chemiluminescence (LucCL) assay when mixed with plasma or whole blood of healthy subjects and hemodialysis patients. However, vitamin C at a physiologic concentration of 100 µmol/L cannot significantly enhance LucCL intensity. Reasons of different vitamin C concentrations, study protocols (i.e., 8 weeks of supplementation vs. a single bolus injection after a dialysis session) and assays for ROS detection (i.e., DCF-DA assay vs. LucCL assay) may possibly account for the variance in vitamin C behavior in different compartments (i.e., intracellular in vivo vs. extracellular in vitro).

hOGG1 and hMTH1 have been identified to be involved in repair of DNA containing the oxidized base of 8-OHdG. hOGG1 removes 8-OHdG from DNA by breaking the glycosidic bond of 8-OHG residue from oxidatively damaged DNA and cleaving the phosphodiester bond at the resulting apurinic site via β elimination [21, 22]. hMTH1 prevents the incorporation of 8-OHG into cellular DNA by hydrolyzing 8-OH-deoxyguanosine triphosphate (dGTP) to 8-OH-deoxyguanosine monophosphate (dGMP) [23, 24]. Several groups have shown that both 8-OHdG levels and its repair are induced in human leukocytes and rat kidney by oxidative stress [49, 50]. Therefore, in addition to analyzing lymphocyte 8-OHdG, it is of interest to assess its repair. In the current study we have demonstrated that hOGG1 is inducible at the mRNA level by hemodialysis in peripheral lymphocytes and its level is higher than those of undialyzed patients and healthy controls. Furthermore, gene expression of hOGG1 is elevated following vitamin C administration for 24 hours and remains higher than the level after placebo treatment in both chronic hemodialysis patients of normal and increased iron stores. Increased mRNA levels of DNA repair gene may not necessarily reflect increased DNA repair enzyme activity. However, investigators have proposed that high hOGG1 mRNA levels are associated with high repair of 8-OHdG [51, 52]. Accordingly, up-regulation of hOGG1 expression by vitamin C seen in the current study may be linked to the increase in 8-OHdG repair enzyme activity, suggesting that the decrease in 8-OHdG levels of lymphocyte DNA may be attributed partially to the activation of hOGG1 after vitamin C supplementation. Cooke et al. [25] disclosed a significant decrease in 8-OHdG levels in peripheral mononuclear cell DNA, followed by a sequential, significant increase in the levels of serum and urine 8-OHdG from healthy subjects undergoing supplementation with 500 mg/day vitamin C. The oxidatively damaged deoxynucleotides generated in the repair processes may subsequently be enzymatically hydrolyzed to stable, water-soluble deoxynucleosides and finally excreted in the urine. The findings of Cooke et al. [25] and in our study suggest that vitamin C promotes the removal of 8-OHdG from DNA via the up-regulation of repair enzymes, and thereby demonstrate a nonscavenging antioxidant effect.

Expression of hMTH1 has been reported in human cancer cell lines [53, 54], indicating upregulation of hMTH1 gene expression in cancer cells with high levels of oxidative stress. In this study we have also found that the mRNA level of hMTH1 maintains higher than the healthy control level. But hMTH1 gene expression is not regulated by vitamin C administration for 24 hours in chronic hemodialysis patients. Our observations suggest that although vitamin C may be important in the up-regulation of hOGG1 gene expression for removal of 8-OHdG from oxidatively damaged DNA in peripheral lymphocytes of chronic hemodialysis patients, it does not appear to play a role in the regulation of hMTH1 gene expression. The difference is unknown and further studies are needed to understand the molecular mechanisms of this regulation.
ACKNOWLEDGMENTS

This study was supported by grants from the National Science Council (NSC 91–2314–B010–073 and NSC 92–2314–B010–027) and Taipei Veterans General Hospital (VGH 91–376–14). The authors are extremely grateful to Miss P.C. Lee for her expert secretarial assistance and graphic design.

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