Decreased Levels of Plasma Vitamin C and Increased Concentrations of Inflammatory and Oxidative Stress Markers After Stroke

Concepción Sánchez-Moreno, PhD; John F. Dashe, MD, PhD; Tammy Scott, PhD; David Thaler, MD, PhD; Marshal F. Folstein, MD; Antonio Martin, MD, PhD

Background and Purpose—Inflammatory response is a critical component of the complex pathophysiological response to stroke. Vitamin C has been shown to have important roles in cell performance and vascular function. In this study, we compared the nutritional status and levels of inflammatory markers between stroke cases and controls and assessed which antioxidant was associated with levels of inflammatory markers and oxidative stress among cases and controls.

Methods—We evaluated the nutritional status and measured plasma levels of vitamins C and E, uric acid, serum levels of C-reactive protein (CRP), the cytokines tumor necrosis factor-α and interleukin-1β, intercellular adhesion molecule-1 (ICAM-1) and chemokine monocyte chemoattractant protein-1 (MCP-1), prostaglandins PGE2 and PG12, and 8-isoprostanes (8-epiPGF2α) for 15 patients with ischemic stroke within 2 to 5 days after stroke onset and for 24 control subjects.

Results—Stroke patients had significantly lower plasma levels of vitamin C than did controls. Among stroke patients, CRP was significantly elevated, as were the ICAM-1, MCP-1, and 8-epiPGF2α, but the prostaglandins PGE2 and PG12 were significantly reduced. Interestingly, vitamin C concentration was significantly inversely correlated with the levels of CRP and 8-epiPGF2α among stroke patients, and 8-epiPGF2α was significantly associated with the levels of CRP. Uric acid was also elevated among stroke patients.

Conclusions—Lower vitamin C concentration, higher serum levels of inflammatory (CRP, ICAM-1, MCP-1) and oxidative stress (8-epiPGF2α) markers, and lower PG12 and PGE2 concentrations among stroke patients indicate the presence of an inflammatory response associated with stroke. (Stroke. 2004;35:163-168.)

Key Words: antioxidants ■ C-reactive protein ■ inflammation ■ oxidative stress ■ stroke

Stroke is the second-leading cause of mortality worldwide.1 In addition, about one quarter of patients suffering stroke are found to be demented 3 months later.2 This percentage represents a risk >9 times that of persons in the general population. If impairment in cognitive status rather than dementia is considered, ≈75% of patients ≥75 years of age and ≈50% of patients <65 years old are affected.3 For those patients who remain cognitively intact a few months after stroke, the risk of developing delayed dementia is 6 times greater than that among persons in the general population in hospital-based studies and 6 to 9 times greater than in population-based studies.

Stroke-mediated release of proinflammatory cytokines and adhesion molecules is a relevant component of the complex pathophysiological response to stroke.4 Inflammatory mediators such as adhesion molecules and chemokines enable leukocytes to adhere to the vascular endothelial cells and to infiltrate inflammatory cells from the circulation into the ischemic brain within hours after ischemic damage. Both macrophages and endothelial cells produce intercellular adhesion molecule-1 (ICAM-1) in response to inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), whereas monocyte chemoattractant protein-1 (MCP-1) expression is sited to endothelial, epithelial cells, and glial cells.5,6

Various cross-sectional studies support the notion that C-reactive protein (CRP) may be a marker for stroke and poststroke status among persons in the US population.7,8 Several studies also support the role of CRP in the prediction of ischemic stroke risk and outcome, as well as the possible role of inflammation before and after stroke.9,10

Antioxidant nutrients have important roles in cell function and have been implicated in processes associated with aging, including vascular, inflammatory, and neurological damage.11 The protective effect of vitamin E (vit-E) against cognitive decline and neurodegenerative disease has been
explore in several epidemiological and clinical studies during the last decade. Vitamin C (vit-C) has been shown to significantly improve endothelium-dependent vasodilation among diabetics and among patients with coronary artery disease, perhaps by reducing excess superoxide production and decreasing the levels of nitric oxide inactivation.

F$_2$-isoprostanes are a family of eicosanoids of nonenzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. Several reports have indicated that isoprostanes are reliable markers of oxidative stress in vivo. One isoprostane, 8-isoprostane ($8$-epiPGF$_{2\alpha}$), has been shown to have biological activity as a potent vasoconstrictor.

A large body of evidence indicates that micronutrient status is an important determinant of vascular dysfunction and contributes to vasculature changes in the brain, risk of stroke, and cognitive impairment among the elderly.16,17 However, the causal relationship between nutrients and the inflammatory response are poorly understood. In this study, we examine the degree of inflammation in relation to nutritional status. Implications of this duality of the posttraumatic inflammatory response for the prevention and/or treatment of brain-injured patients are discussed.

**Subjects and Methods**

**Subjects**

Using the scanning protocol developed and implemented by the New England Medical Center (Boston, Mass), we enrolled 15 ischemic stroke patients and 24 control subjects from the stroke service inpatient and outpatient population of the center. The study was approved by the Institutional Review Board, reference number HIRC-5515. We used acute neuroimaging studies (CT and MRI) and neuropsychological studies (MMSE and trail making) to confirm and classify stroke subtype and localization. The inclusion criteria for cases and controls were nonischemic type of stroke; presence of symptomatic intracranial tumor, infection, inflammation, or other nonischemic cause of neurological dysfunction; moderate to severe aphasia; presence of bilateral blindness or deafness; history or current diagnosis of alcohol or substance abuse; history of mental retardation; presence of encephalopathy or delirium; and unwillingness to give consent. Blood was collected 48 to 120 hours after stroke.

The goal of this study was to compare the nutritional status and levels of inflammatory markers between stroke cases and controls. We assessed the nutritional status for 3 months before stroke (using the Food Frequency Questionnaire [FFQ] as a measure of prestroke nutritional status for the cases), determined the serum antioxidant levels (after stroke for the cases), and evaluated the levels of inflammatory markers (after stroke for the cases). The rationale for these determinations was to assess which antioxidant levels may have followed the stroke and which nutrient was associated with levels of inflammatory markers and oxidative stress among cases and controls.

**Nutritional Status Determination**

Dietary history data were obtained through the FFQ, which includes 125 food items, from both cases (48 to 120 hours after stroke) and controls on the day that blood was drawn. The reference period for the FFQ was the preceding 3 months.

**Cognitive Evaluation**

We use the Mini-Mental State Examination to determine the cognitive status of the stroke patients. The test was administered on the day that blood was drawn.

**Quantification of Vit-E and Vit-C**

**Vitamin E**

Tocopherol content of plasma was measured by reverse-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection. Tocopherol concentration was expressed in micromoles per liter.

**Vitamin C**

Ascorbate was analyzed by paired-ion, reversed-phase HPLC coupled with electrochemical detection. Ascorbate concentration was expressed in micromoles per liter.

**Quantification of Uric Acid**

We analyzed uric acid by paired-ion, reversed-phase HPLC coupled with electrochemical detection using the same procedure described for vit-C determination with the electrode potential of 0.6 V but with the gain set at 1 $\mu$amp.

**Quantification of CRP**

We used ELISA to determine soluble levels of CRP with an ultrasensitive enzyme-linked immunosorbent assay (ELISA; Abbott Laboratories).

**Determination of TNF-α and IL-1β**

Plasma levels of TNF-α and IL-1β were determined by means of an ELISA. Precoated goat anti-rabbit antibodies were used to capture the specific cytokines TNF-α and IL-1β with a 96-well plate (ELISA plate) of polycloncal cytokine antibody and sample/standard. TNF-α and IL-1β concentrations for each sample were determined by plotting the unknown samples against the calibration curve and by correcting for the dilution factor.

**Quantification of ICAM-1 and MCP-1**

We used ELISA to determine soluble levels of ICAM-1 and MCP-1 proteins. We used antibodies directed to bind human ICAM-1 or MCP-1 (clones E1/6 and LB-2) from Becton Dickenson Cellular Imaging Systems. ICAM-1 and MCP-1 concentrations for each sample were determined by plotting the unknown samples against the calibration curve and by correcting for the dilution factor.

**Determination of PGE2 and PGI2**

To measure prostaglandins, we used a high-sensitivity immunoassay based in a competitive binding technique in which prostaglandins present in a sample compete with a fixed amount of alkaline phosphatase–labeled prostaglandins for sites on a mouse monoclonal antibody. During incubation, the mouse monoclonal antibody becomes bound to the goat antimouse antibody coated onto the microplate wells. After a wash to remove excess conjugate and unbound sample, we added a substrate solution to the wells to determine the bound enzyme activity. Color intensity was therefore inversely proportional to the concentration of the PGE2 or PG12 measured in the sample.

**Quantification of 8-epiPGF$_{2\alpha}$**

We used an enzyme immunoassay kit (Cayman Chemical) to determine the concentration of 8-epiPGF$_{2\alpha}$ in plasma, as we have previously reported.
Statistical Analysis
We compared concentration of nutrients and levels of inflammatory markers between groups by 1-way analysis of variance using the Systat 10 program. We used Student’s t tests to detect differences between cases and controls. Within each group, we used linear regressions to examine the relationship between the different markers of inflammation and the antioxidant nutrients vit-E and vit-C. Significance was accepted if the null hypothesis was rejected at the $P<0.05$ level.

Results
In Table 1, we present a general description of the cases’ and controls’ clinical characteristics and medical history.

Nutritional status and micronutrient intake of cases and controls were assessed with the FFQ (19) that was filled out by the study participants with the help of their families. Caloric intake among the participants in both groups was similar. Vit-C intake was also similar among cases and controls (84 versus 101 mg/d). However, we noted that cases had a lower intake of several B-vitamins (B6, B12, pantothenic acid, niacin, and riboflavine; $P<0.05$) than controls.

Cognitive status of the stroke patients was determined with the Mini-Mental State Examination. Using the recommended cognitive guideline scores ($\geq 21$ = mild cognitive impairment, 10 to 20 = moderate impairment, and $\leq 9$ = severe impairment), we found that most stroke patients showed mild cognitive impairment (mean score, 26.40±4.52) 48 to 120 hours after stroke.

Several differences between cases and controls were observed (Table 2). Several correlations among vit-C, inflammatory markers, and prostaglandins are summarized in Table 3. Stroke patients had significantly lower ($P=0.003$) plasma vit-C levels than did controls (39±6 versus 61±4 mg/dL; Table 2). Interestingly, vit-C levels among stroke patients were significantly negatively correlated with CRP concentration ($r=-0.53$, $P=0.04$) and positively associated with $\alpha$-tocopherol ($r=0.54$, $P=0.03$; Table 3).

Plasma concentration of $\alpha$- and $\gamma$-tocopherol was not significantly different between stroke cases and controls (Table 2). Uric acid levels were increased, but not significantly elevated, among stroke patients (331.1±124.9 mol/L) compared with controls (303.4±71.4 mol/L). Stroke patients had significantly higher ($P=0.03$) CRP levels than did controls. Serum levels were 10.4±3.4 mg/L for stroke patients and 1.9±0.2 mg/L for controls (Table 2).

For participants in the control group, we found that plasma $\alpha$-tocopherol levels were significantly and inversely correlated with CRP levels ($r=-0.44$, $P=0.03$; Table 3). This finding may be relevant because CRP levels have been found to be highly associated with risk of stroke. IL-1$\beta$ concentration was significantly associated with PGI2 levels ($r=0.16$, $P=0.02$; Table 3).

We also found that serum levels of MCP-1 were significantly elevated among participants in the stroke group (153±10 pg/mL) compared with controls (120±4 pg/mL; $P=0.003$; Table 2). An element of the inflammatory response common to several injuries is the recruitment of monocytes into tissues, which is mediated in part by MCP-1.

Stroke patients had significantly lower plasma PGI2 (802±107 pg/mL) than did controls (1969±133 pg/mL; $P<0.0001$; Table 2). Plasma PGE2 levels were also significantly lower among stroke cases (170±13 pg/mL) than controls (208±11 pg/mL; $P=0.02$) (Table 2). Interestingly, among control subjects, we observed an inverse correlation between $\alpha$-tocopherol concentration and levels of PGE2.

<p>| TABLE 1. Demographic and Clinic Characteristics of Stroke Cases and Controls |
|-------------------------------------|---------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Age, y</th>
<th>Cases</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Hematocrit</td>
<td>67±12 (60–91)</td>
<td>61±10 (60–89)</td>
</tr>
<tr>
<td>Serum albumin, g/L</td>
<td>4.4±0.7 (3.1–5.7)</td>
<td>4.8±0.3 (4.4–5.5)</td>
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<tr>
<td>Total protein, g/L</td>
<td>7.3±1.0 (5.4–9.0)</td>
<td>7.8±0.4 (7.2–8.7)</td>
</tr>
<tr>
<td>Creatinine, μmol/L</td>
<td>97.2±53.04 (53.04–274.04)</td>
<td>88.4±17.68 (53.04–114.92)</td>
</tr>
<tr>
<td>Uric acid, μmol/L</td>
<td>333.1±124.9 (148.7–493.7)</td>
<td>303.4±71.4 (172.5–422.3)</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>7.55±2.83 (5.11–14.04)</td>
<td>5.06±0.60 (4.44–6.60)</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.75±0.88 (3.21–6.32)</td>
<td>5.23±0.96 (3.72–8.09)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.03±1.30 (0.71–5.65)</td>
<td>1.32±0.64 (0.54–2.87)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.17±0.40 (0.60–2.10)</td>
<td>1.13±0.33 (0.65–2.10)</td>
</tr>
<tr>
<td>Total homocysteine, μmol/L</td>
<td>10.1±4.7 (4.8–17.9)</td>
<td>ND</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein. Values are mean±SD (range).

<table>
<thead>
<tr>
<th>TABLE 2. Variables Evaluated in Stroke Cases and Controls</th>
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<tbody>
<tr>
<td>Cases</td>
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<tr>
<td>------</td>
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<tr>
<td>Vit-C, μmol/L</td>
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<tr>
<td>$\alpha$-Tocopherol, μmol/L</td>
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<tr>
<td>$\gamma$-Tocopherol, μmol/L</td>
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<tr>
<td>Uric acid, μmol/L</td>
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<tr>
<td>CRP, mg/L</td>
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<tr>
<td>ICAM-1, ng/mL</td>
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<tr>
<td>MCP-1, pg/mL</td>
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<td>PGI2, pg/mL</td>
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<tr>
<td>PGE2, pg/mL</td>
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<tr>
<td>TNF-α, pg/mL</td>
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<tr>
<td>IL-1$\beta$, pg/mL</td>
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<tr>
<td>8-epiPGF2α, pg/mL</td>
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Values are mean±SEM.
Elevated PGF2α concentration has been associated in the elderly with unfavorable effects on the immune system. Our results reinforce previous findings on the potential benefit to the immune system of increasing vit-E intake.

Serum levels of cytokines (TNF-α and IL-1β) were lower among stroke patients (6.0 ± 0.4 and 0.5 ± 0.05 pg/mL, respectively) than among controls (7.0 ± 0.2 and 1.5 ± 0.3 pg/mL; Table 2), perhaps because the blood was collected 48 to 120 hours after stroke. At this time, cytokine expression declines to basal or below basal levels after its maximum increase within 24 hours after stroke. Thus, as other investigators have reported, the inflammatory cascade that occurs after stroke is accompanied by the release of several inflammatory markers, but the course of secretion of these chemokines is different for each molecule.

Plasma levels of isoprostanes (8-epiPGF2α) were significantly higher among stroke patients (198 ± 14 pg/mL) than controls (166 ± 4 pg/mL; P = 0.02; Table 2). The F2-isoprostanes, also referred to as 8-epiPGF2α, have received significant attention because of their vasoconstrictive platelet activation and mitogenic properties. Interestingly, we observed an inverse correlation between plasma vit-C concentration and 8-epiPGF2α levels (r = -0.52, P = 0.05) among stroke patients and a positive association between 8-epiPGF2α and CRP concentrations (r = 0.65, P = 0.008; Table 3).

Discussion

In this study, we have shown that ischemic stroke cases had lower levels of plasma vit-C and higher levels of inflammatory markers than controls while having similar vit-C intakes and levels of inflammatory markers. Plasma levels of vit-C were significantly inversely correlated with CRP levels. This finding suggests that vit-C may play a critical role in the stroke-mediated inflammatory response and may be associated with neurological changes and cognitive impairment.

The mechanisms involved in the reduction in vit-C levels after stroke remain unknown. Interestingly, we did not observe significant differences in total vit-C intake between groups, but plasma levels of vit-C were significantly lower among cases. Our results are consistent with other observations in different animal models in which antioxidant depletions were observed after focal cerebral ischemia brain injury. Several studies have reported increased mortality rates from stroke in individuals with low vit-C concentrations. A number of factors may be responsible for this phenomenon, including the physiological need of glial cells, particularly astrocytes, coupled with the removal of increased levels of glutamate after stroke. Another factor may be the formation of free radicals after stroke.

Inflammatory markers such as CRP, adhesion molecules, and cytokines are among the most relevant molecules expressed after stroke. In our study, we found that stroke patients had significantly elevated CRP levels, which were inversely associated with vit-C concentration and positively associated with 8-epiPGF2α (Table 3). Several prospective studies have shown that elevated serum CRP concentration is a strong predictor of cardiovascular events, including stroke. CRP plays an important role as a marker of outcome and may determine the degree of recovery for stroke patients. Interestingly, ≈70% of controls had CRP levels >1 mg/L, considered the clinical upper limit of normal. Elevated CRP concentration has been associated with a 2-fold increase in risk of ischemic stroke. The significant inverse association between α-tocopherol and CRP and the positive association between vit-C and α-tocopherol suggests that increasing vit-E and vit-C concentrations may decrease the risk of cardiovascular accidents in the elderly and may help to explain the beneficial effects of these nutrients in the cardiovascular system.

Our findings also showed elevated concentrations of ICAM-1 and MCP-1 in stroke patients. ICAM-1 plays a critical role in mediating cell-cell contact between leukocytes and cells of various origins. Although it is weakly expressed in nonstimulated conditions, ICAM-1 expression is upregulated by inflammatory cytokines and appears to be another critical player in the poststroke inflammatory response. Serum levels of ICAM-1 were significantly greater among stroke patients (266 ± 17 ng/mL) than controls (221 ± 7 ng/mL; P = 0.04; Table 2). This finding further supports the importance of the inflammatory response in stroke. IL-1β induces capillary endothelial cells to secrete chemokines such as MCP-1 and adhesion molecules such as ICAM-1. Recent reports have shown increased levels of TNF-α and IL-1β within 24 hours after stroke. Because we collected blood between 48 and 120 hours after stroke, we did not see higher levels of cytokines. Rather, we observed concentrations that were below basal levels. Cytokines are involved in the induction of PGI2 synthesis in human vascular endothelial cells. We found a positive association between IL-1β and PGI2 in controls (Table 3).

The differences in the degree of inflammatory response suggest that the type of poststroke response may play a key role in determining the extent of cognitive impairment and a patient’s prognosis. Another important source of molecules is the eicosanoids, a group of bioactive compounds derived from arachidonic acid metabolism via enzymatic pathways such as cyclooxygenase and lipoxygenase. These compounds play diverse roles in brain physiology and are altered by stroke. For example, PGI2 protects against posts ischemic brain injury. PGI2 is a potent vasodilator and inhibitor of
platelet aggregation, leukocyte activation, and leukocyte-endothelial interactions. Recent studies have shown that prostaglandins have important roles as vasodilators and in preventing clot formation.\textsuperscript{40} However, the use of cyclooxygenase-2 inhibitors, a major source of PGII2 in normal subjects,\textsuperscript{41} may lead to increased cardiovascular events and poor outcomes after stroke. We observed a significant reduction in both PGII2 and PGE2 concentrations among stroke patients compared with controls.

Oxidative stress in association with stroke has been discussed in various studies.\textsuperscript{42,43} We evaluated the presence of metabolites derived from prostaglandins but formed by random oxidation of tissue phospholipids by oxygen radicals. These compounds, considered markers of oxidative stress, have been found to be potent vasoconstrictors and to modulate platelet aggregation.\textsuperscript{32} In our study, we found that plasma vit-C was inversely correlated with 8-epiPGF\textsubscript{2\alpha} concentration among stroke patients. We also observed that 8-epiPGF\textsubscript{2\alpha} levels were highest among stroke patients who smoked and that vit-C concentration was lowest for these patients. Other investigators have reported similar observations.\textsuperscript{44,45}

As a case-control study, this investigation has limitations, including the impossibility of assessing temporality. Therefore, we cannot assess with complete certainty that the poststroke levels observed in antioxidant nutrients and inflammatory markers were caused by the stroke itself because we do not have prestroke blood information. However, we have data from the FFQ that suggest that these changes were mediated by stroke. Another limitation of this study is the FFQ; recall of diet over the previous 3 months may differ between cases and controls.

When these findings are put together, our study strongly suggests that early vit-C depletion may play a critical role in brain injury. Thus, evaluating the effects of vit-C supplementation for stroke patients has promise in the treatment of stroke.

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