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Plasma Levels of Antioxidant Vitamins, Selenium, Total Sulfhydryl Groups and Oxidative Products in Ischemic-Stroke Patients as Compared to Matched Controls in Taiwan

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The possible involvement of oxidative damage and antioxidant protection has been suggested in the pathogenesis of stroke which is the second-leading cause of death in Taiwan. In this study we investigated the relationship between ischemic stroke and plasma status of antioxidants and oxidative products. Plasma levels of vitamin A, α-tocopherol, carotenoids, selenium (Se), total SH groups (T-SH), thiobarbituric acid-reactive substances (TBARS) and protein carbonyl, a marker of protein damage, were determined in ischemic-stroke patients (n = 36, blood sampled within 24 hrs after the clinical event) in comparison with 21 matched controls. The cholesterol-adjusted carotenoids and vitamin E were significantly lower ($P < 0.05$) in the plasma of ischemic-stroke patients than those of the controls. TBARS were higher ($P < 0.05$) in the patients than in the controls but Se, T-SH and protein carbonyls were not significantly different between the two groups. Sepration of the patients into small-artery ischemic stroke (SAIS, n = 17) and large-artery ischemic stroke (LAIS, n = 19) groups revealed that both carotenoids/cholesterol and vitamin E/cholesterol ratios were significantly lower in both LAIS and SAIS groups than the controls (n = 21) while vitamin A/cholesterol was not different among the three groups. TBARS were only significantly higher in the LAIS group. The results demonstrated that, within 24 hrs after the clinical event, the acute-ischemic stroke patients had lowered levels of cholesterol-adjusted carotenoids and α-tocopherol but elevated levels of TBARS in the plasma as compared to the matched controls. It remains to be resolved as to whether enhanced lipid peroxidation is a cause or a result of lowered antioxidants in ischemic stroke.

Keywords: Ischemic stroke, plasma, antioxidant vitamins, oxidative stress

INTRODUCTION

Stroke, or cerebrovascular disease, is the third leading cause of death in the U.S.[1] and the second in Taiwan.[2] Accumulating evidence indicates that reactive oxygen species such as hydroxyl radicals, superoxide anions, singlet oxygen and nitric oxide play an important role in aging and disease including cancer, cardiovascular disease and stroke,[3,4] and that antioxidants are important in protecting against oxidative damage.[5,6] A num-

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ber of animal studies demonstrate that oxidative damage to membrane lipids and proteins is potentiated by ischemia and reoxygenation leading to cell and tissue damage[7–11] and that levels of lipid-soluble antioxidants including α-tocopherol and ubiquinones are decreased during the ischemia/reperfusion process.[12]

Several studies have suggested that increased consumption of fruits and vegetables[13–15] and *antioxidant* vitamins[16] is associated with reduced risk of stroke.[13–15] A nutrition intervention trial in Linxian, China[17] demonstrated that in males cerebrovascular mortality is decreased by 55% (RR = 0.45, $P < 0.05$) among individuals receiving multiple vitamin/mineral supplements. However, except for a few epidemiological studies which have revealed that poor plasma status of vitamin C and β-carotene is associated with increased mortality from stroke,[18,19] little information exists regarding the relationship between ischemic stroke and plasma or serum status of antioxidants and oxidative products. In this study we, therefore, compared the plasma levels of vitamin A, vitamin E, carotenoids and thiobarbituric acid-reactive substances (TBARS), protein carbonyls and total SH groups of acute ischemic-stroke patients with those of healthy subjects. Selenium (Se), an integral part of the enzyme glutathione peroxidase,[3] was also determined because a 9-year follow-up study in the Netherlands has shown that serum Se levels were decreased in patients who died of stroke.[20]

PATIENTS AND METHODS

We recruited the patients who were admitted to the Department of Neurology at Chi-Mei Foundation Hospital between July 1995 and March 1996, and who satisfied two criteria: 1) neurological deficit due to acute ischemia in the territory of the carotid artery or basilar artery, and 2) sudden focal neurological deficit that lasting more than 24 hrs. Diagnosis was based upon clinical examination by neurologists, electroencephalogram (EEG) and brain computed tomography performed on admission. Patients (Table I, $n = 36$) were further divided into the small-artery ischemic stroke (SAIS, $n = 17$) group and the large-artery ischemic stroke (LAIS, $n = 19$) group according to EEG and brain computed tomogram. SAIS was defined as vascular insult confined to the territory of the deep arteries with the brain computed tomography showing lacunar infarcts and normal or mildly normal EEG, and the LAIS as vascular insult that occurs outside the territory of deep perforating arteries. All patients received similar antiplatelet therapy (150 mg aspirin orally per day) and supportive care (light hydration, nursing, and rehabilitation). The sex- and age-matched control subjects (Table I) were those who visited Chi-Mei Foundation Hospital for health examination during the same period of time and who had no abnormalities (including diabetes mellitus and other neurologic disorders)

TABLE I Basic information about the patients with large-artery ischemic stroke (LAIS) or small-artery ischemic stroke (SAIS) and of the matched controls

		Ischemic stroke		
	Control	Total	LAIS	SAIS
Number of subjects	21	36	19	17
Sex (♂/♀)	8/13	14/22	7/12	7/10
Age (mean ± SD)	61 ± 5	67 ± 11	69 ± 11	67 ± 10
Age range	55 – 70	44 – 88	54 – 88	44 – 82
Cholesterol (mg/dL)	211 ± 59	208 ± 38	204 ± 35	212 ± 42
Hemoglobin (mg/dL)	14.3 ± 1.5	13.9 ± 1.6	14.1 ± 1.7	13.7 ± 1.5
Albumin (mg/dL)	4.14 ± 0.35	3.84 ± 0.30	3.82 ± 0.30	3.87 ± 0.31

Values are means ± SD. There are no significant differences in the age or in the levels of cholesterol, hemoglobin and albumin either between the controls and the total patients, or among the control, LAIS and SAIS groups.

found in the examination. We used a questionnaire to survey nutrient supplement habit of both the controls and patients (their family members) and those who took vitamin supplements regularly were excluded. The patients we recruited had approximately the same ages but had a female to male ratio of approximately 2:3. Because the controls (a total of 99 were originally recruited) tended to be younger than the patients, those who were below 55 years old (most volunteers happen to be below this age) or were suspected to have taken vitamin supplements were excluded. Among the 21 subjects selected the male to female ratio was roughly equal to that of the patients. The study was approved by an institutional review committee and all subjects gave informed consent.

Preparation of Plasma

Venous blood was drawn from the ischemic-stroke patients between 12 and 24 hrs after onset of stroke into vacutainers containing EDTA. After centrifugation at 1,500 g, 4° for 10 min, plasma was frozen at –20° and shipped under frozen state by express mail to the Vitamin Laboratory at the National Chung-Hsing University for measurements. Plasma samples were stored for no more than five days at –20° before analysis (except for selenium), during which there were no changes in the stability of various assays.

Biochemical Assays

Plasma was thawed at 37° for 3 min and placed in an ice bath for no more than 2 hours before the last assays began. Vitamin A (as retinol) and vitamin E (as α-tocopherol) were measured simultaneously by HPLC essentially as described previously.[21] We adopted the modified procedure by Gunter *et al.*[22] by adding ascorbic acid to the extraction medium (but not during storage) to avoid degradation of vitamin E by lipid peroxides that could build up during long-term storage. Carotenoids rather than β-carotene alone were measured in this study. Plasma (0.5 mL) was extracted with ethanol followed by petroleum ether before centrifugation at 4,000 g for 5 min. The absorbance of the upper (petroleum ether) layer was measured and the concentration of carotenoids was calibrated with β-carotene.[23]

T-SH were measured using Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) as described previously.[24] Protein carbonyls were measured using 2,4-dinitrophenylhydrozene as described previously.[25] TBARS were measured following precipitation of proteins, lipoproteins, bilirubin and neutral lipids using TCA/HCl as described previously.[26] Plasma concentration of Se was determined by a fluorimetric method as described elsewhere.[27] Plasma contents of cholesterol, triglycerides, hemoglobin and albumin were determined on fresh plasma using an automatic analyzer (Kodak 341-4379).

Data, expressed as means ± SD, were analyzed using the Mann-Whitney U test (for two-group comparison) or the Kurskal-Wallis nonparametric ANOVA (for three-group comparison) followed by the Mann-Whitney U test for group mean comparisons. A P value < 0.05 was considered statistically significant.

RESULTS

Table I shows the basic information about the patients and matched controls. Plasma cholesterol (a relatively minor risk factor for stroke)[15] was similar between the controls and patients. The light hydration treatment (1.5 L saline/d) intended to maintain fluid balance did not lead to hemodilution, as evidenced by essentially the same levels of hemoglobin and albumin between the patients and controls.

As compared to the controls, the patients had significantly higher plasma levels of TBARS but lower levels of vitamin A, carotenoids and α-tocopherol, while Se, T-SH and protein carbonyls were not different between the two

TABLE II Plasma levels of fat-soluble antioxidants, total SH groups (T-SH), selenium (Se), and oxidative products and ratios of fat-soluble antioxidants to cholesterol in ischemic-stroke patients as compared to matched controls

	Control ($N = 21$)	Ischemic stroke ($N = 36$)	P
Vitamin A (μmol/L)	2.59 ± 0.90	2.09 ± 0.84	0.04
Vitamin A/cholesterol (μmol/mmol)	0.48 ± 0.14	0.40 ± 0.19	0.09
Vitamin E (μmol/L)	31.8 ± 17.7	21.6 ± 7.7	0.02
Vitamin E/cholesterol (μmol/mmol)	5.7 ± 2.5	4.1 ± 1.5	0.01
Carotenoid (μmol/L)	4.2 ± 1.9	2.4 ± 1.3	0.001
Carotenoid/cholesterol (μmol/mmol)	0.85 ± 0.55	0.46 ± 0.24	0.007
T-SH, μmol/L	344 ± 48	348 ± 53	0.78
Se, μmol/L	2.9 ± 0.7	2.7 ± 0.9	0.43
TBARS, μmol/L	0.71 ± 0.30	0.89 ± 0.23	0.03
Protein carbonyls μmol/L	13.6 ± 2.1	14.0 ± 2.9	0.56

Data, expressed as means ± SD, are analyzed using the Mann-Whitney U test. P values < 0.05 are considered statistically significant.

groups (Table II). The plasma level of vitamin E (1.37 ± 0.76 mg/dL or 32 μmol/L) of our matched controls is somewhat higher than that (1.05 ± 0.47 mg/dL or 24 μmol/L, $N = 99$) of Chinese population in Taiwan reported almost 20 years ago.[28] Although we have no reference values of plasma vitamin A for the Chinese population in Taiwan, the values we obtained for our controls (74 ± 26 μg/dL) and patients (60 ± 24 μg/dL) are well within the normal range reported for other ethnic populations, e.g. 40–100 μg/dL in the U.K.[29]

Separation of patients into SAIS and LAIS groups revealed that the LAIS group, but not the SAIS group, had lower ($P < 0.05$) vitamin A level than the control while both the LAIS and SAIS groups had significantly lower levels of carotenoids and vitamin E than the control (Table III). Adjustment of the lipid-soluble vitamins with cholesterol revealed that vitamin A (Fig. 1) was not different among the three groups while carotenoids (Fig. 2) and vitamin E (Fig. 3) were lower ($P < 0.05$) in both LAIS and SAIS groups than the control. The plasma TBARS were higher in both SAIS and LAIS groups than those in the controls but the difference was only significant between the LAIS group and the controls (Fig. 4). The plasma levels of TBARS of our patients and matched controls are comparable to those (0.4 to 1.0 μM) reported by others.[26]

TABLE III Plasma levels of fat-soluble antioxidants and TBARS and ratios of fat-soluble antioxidants to cholesterol in patients with large-artery ischemic stroke (LAIS) and small-artery ischemic stroke (SAIS) as compared to matched controls

	Control ($N = 21$)	LAIS ($n = 19$)	SAIS ($N = 17$)
Vitamin A, μmol/L	2.59 ± 0.90^{a}	1.93 ± 0.75^{b}	2.26 ± 0.92^{ab}
Vitamin A/cholesterol (μmol/mmol)	0.48 ± 0.14	0.37 ± 0.17	0.45 ± 0.23
Carotenoid, μmol/L	4.2 ± 1.9^{a}	2.7 ± 1.2^{b}	2.1 ± 1.3^{b}
Carotenoid/cholesterol (μmol/mmol)	0.85 ± 0.55^{a}	0.48 ± 0.16^{b}	0.40 ± 0.24^{b}
Vitamin E, μmol/L	31.8 ± 17.7^{a}	21.6 ± 8.6^{b}	21.5 ± 6.7^{b}
Vitamin E/cholesterol (μmol/mmol)	5.7 ± 2.5^{a}	4.1 ± 1.7^{b}	4.0 ± 1.3^{b}
TBARS, μmol/L	0.71 ± 0.30^{a}	0.92 ± 0.25^{b}	0.85 ± 0.19^{ab}

Data (as means ± SD) are analyzed using the Kurskal-Wallis nonparametric ANOVA followed by the Mann-Whitney U test for group mean comparisons. Values not sharing the same superscript letters are significantly different ($P < 0.05$).

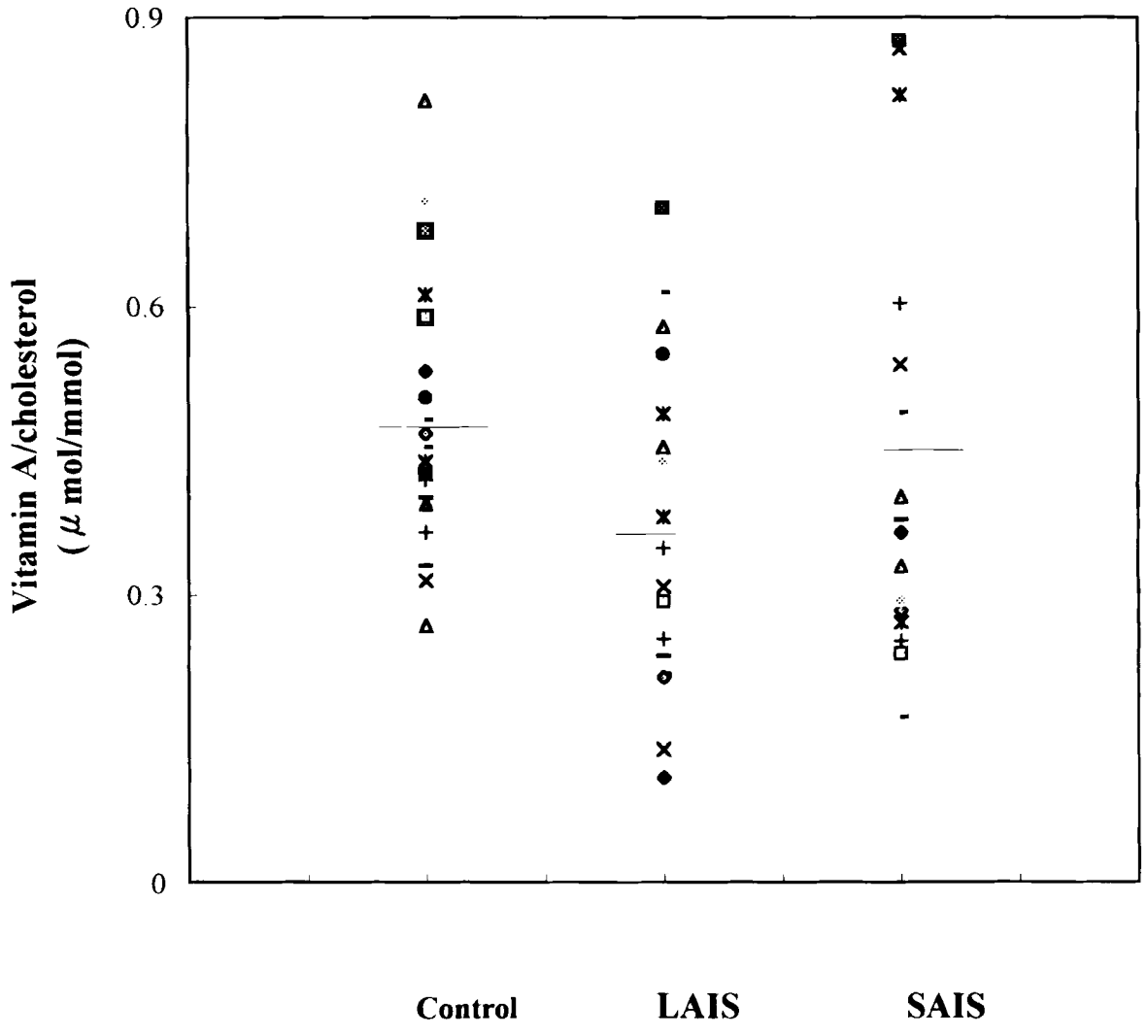

FIGURE 1 Distribution of cholesterol-adjusted vitamin A in the plasma of the controls (n = 21) and patients with large-artery ischemic stroke (LAIS, n = 19) and small-artery ischemic stroke (SAIS, n = 17). Each symbol represents one subject; the lines denote the mean values (SD values are given in Table III) which are significantly different ($P < 0.05$) from each other when followed by different letters.

DISCUSSION

This study aimed to examine the hypothesis derived from animal studies that lipid peroxidation is enhanced and vitamin E is oxidized during cerebral ischemia/reperfusion. We found that the ischemic-stroke patients had lower ($P < 0.05$) plasma levels of vitamin A, carotenoids and vitamin E and lower ($P < 0.05$) ratios of carotenoids and vitamin E to cholesterol than had the controls. Preliminary observations suggest that plasma vitamin C levels may also be lower in the patients than the controls (data not shown). Although it is not clear whether the levels of vitamins in human plasma truly reflect those of cerebral tissues, the present findings are generally in accord with those of the animal studies[9,12,30–32] which showed that vitamin E is decreased at the end of ischemia and with the epidemiological studies by Gey *et al.*,[18,19] which showed that poor plasma status of vitamin

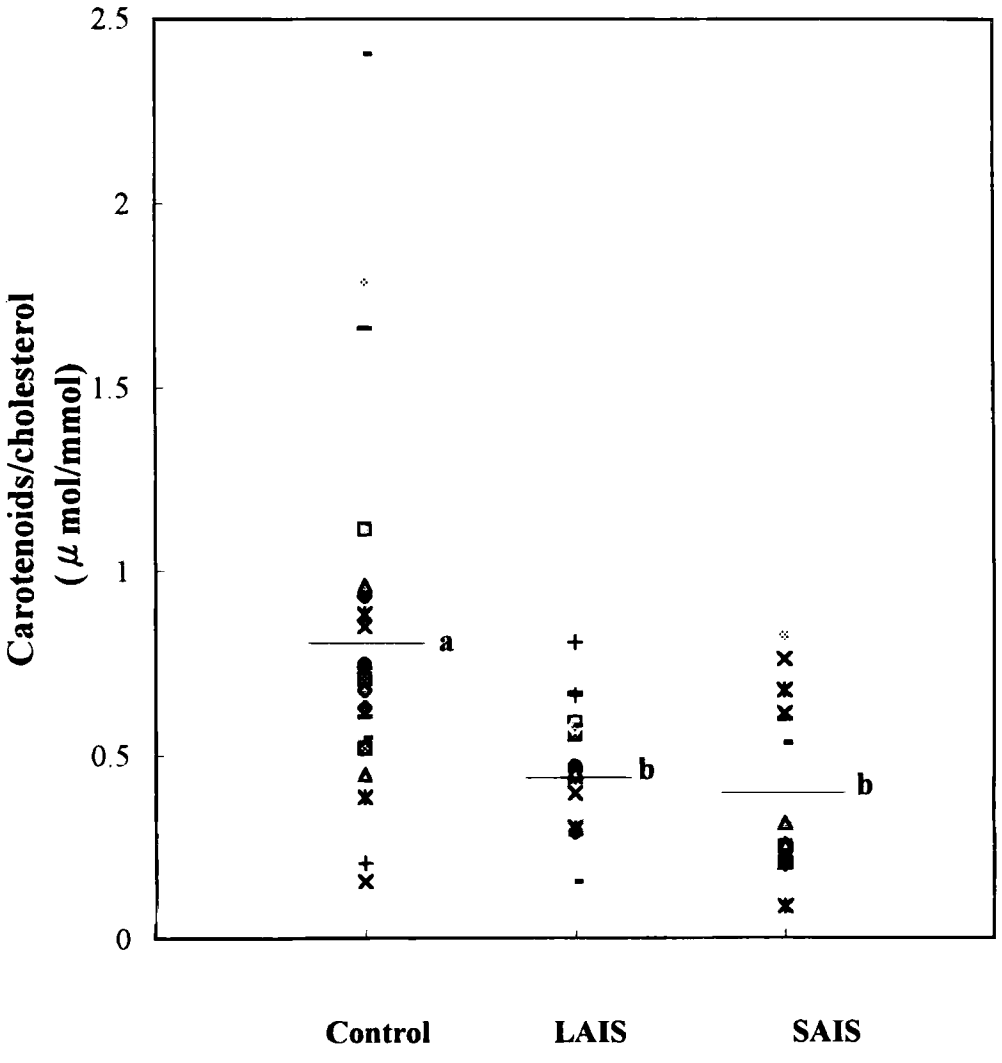

FIGURE 2 Distribution of cholesterol-adjusted carotenoids in plasma of the controls ($n = 21$) and patients with large-artery ischemic stroke (LAIS, $n = 19$) and small-artery ischemic stroke (SAIS, $n = 17$). Symbols and bars are the same as those described in Figure 1.

C and β-carotene is associated with higher mortality from ischemic stroke. The vitamin E level (21.6 μmol/L) of our ischemic-stroke patients is within the normal range (15–40 μmol/L) reported for a US population.[33] That our patients are not deficient in vitamin E and vitamin A (including carotenoids) suggests that the lower levels of these antioxidants in the patients are a result of oxidative stress associated with ischemic stroke rather than a cause of oxidative stress. However, the vitamin E/cholesterol ratio (4.1 μmol/mmol) of our patients is near that (< 4.1 μmol/mmol) reported to be moderately associated with increased risk of cardiovascular death.[34] As we do not have the pre-stroke status of vitamin E (or other antioxidant vitamins) of the patients, it is unclear whether the patients had low plasma vitamin E prior to the onset of ischemic stroke. The average daily intake of vitamin E (10.7 mg α-tocopherol equivalent)[35] in the people of Taiwan appears to be adequate (as compared to that of US RDA), but may not be sufficient to prevent cerebrovascular

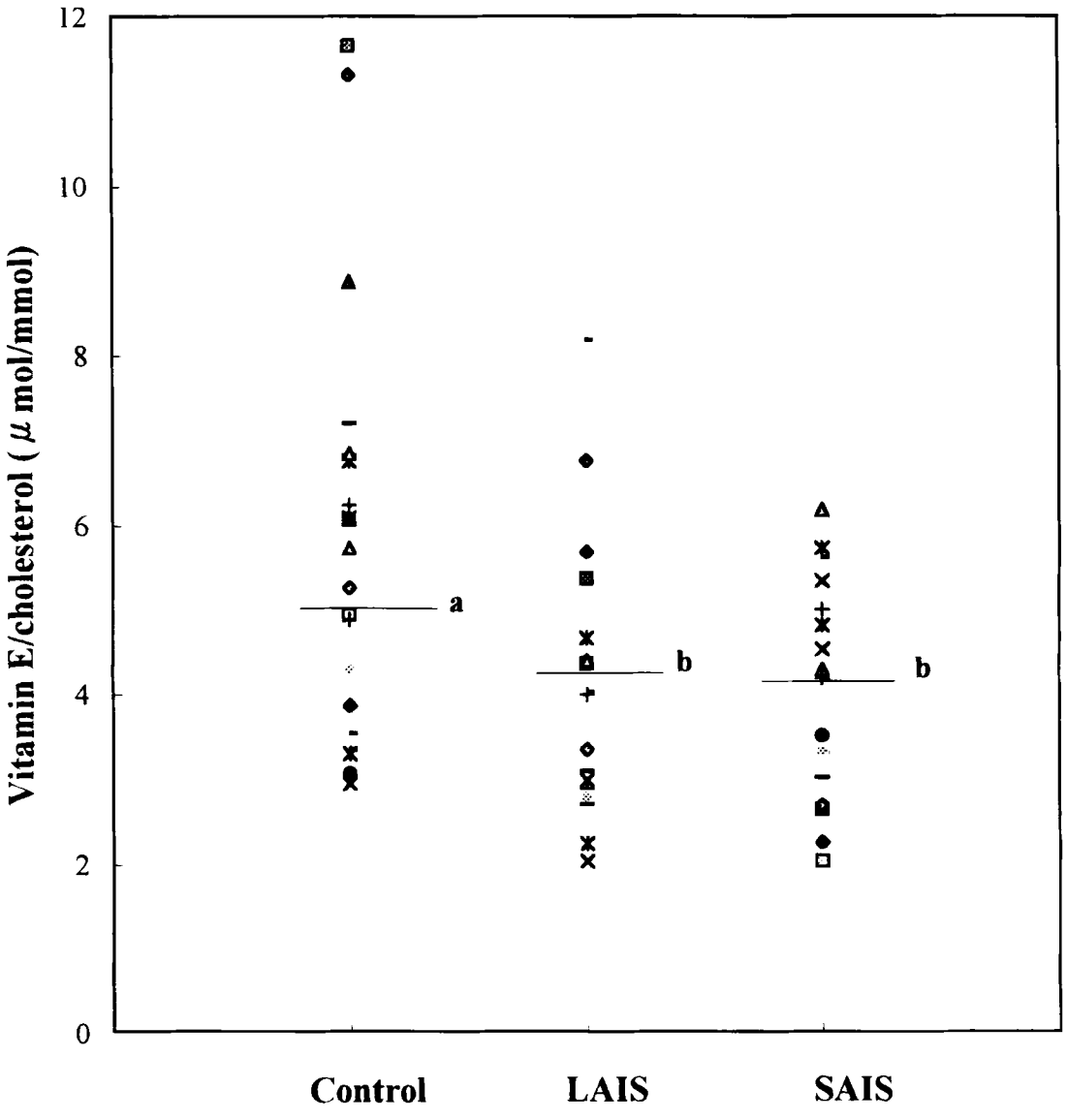

FIGURE 3 Distribution of cholesterol-adjusted vitamin E in the plasma of the controls ($n = 21$) and patients with large-artery ischemic stroke (LAIS, $n = 19$) and small-artery ischemic stroke (SAIS, $n = 17$). Symbols and bars are the same as those described in Figure 1.

disease because the main source of vitamin E is frying oils (mainly soybean oil[35,36] which is rich in unsaturated fatty acids) which may increase the need for vitamin E. Moreover, the relatively high daily salt intake in Taiwan (15 g)[35] may predispose people to high blood pressure (the ninth leading cause of death in Taiwan)[2] and stroke.

The TBA assay is known to have poor specificity. For instance, published mean levels of TBARS in human plasma by either the colorimetric or fluorimetric assay ranged from 4 to 47 μM,[37,38] while the levels obtained by the HPLC-based TBA assay were much lower (1.4 to 0.6 μM).[38] Using an improved HPLC-based assay for TBARS, Chirico *et al.*[39] demonstrated that levels of plasma TBARS in hyperlipidemic patients were elevated, and the absolute levels were between 0.1 μM for the control subjects and

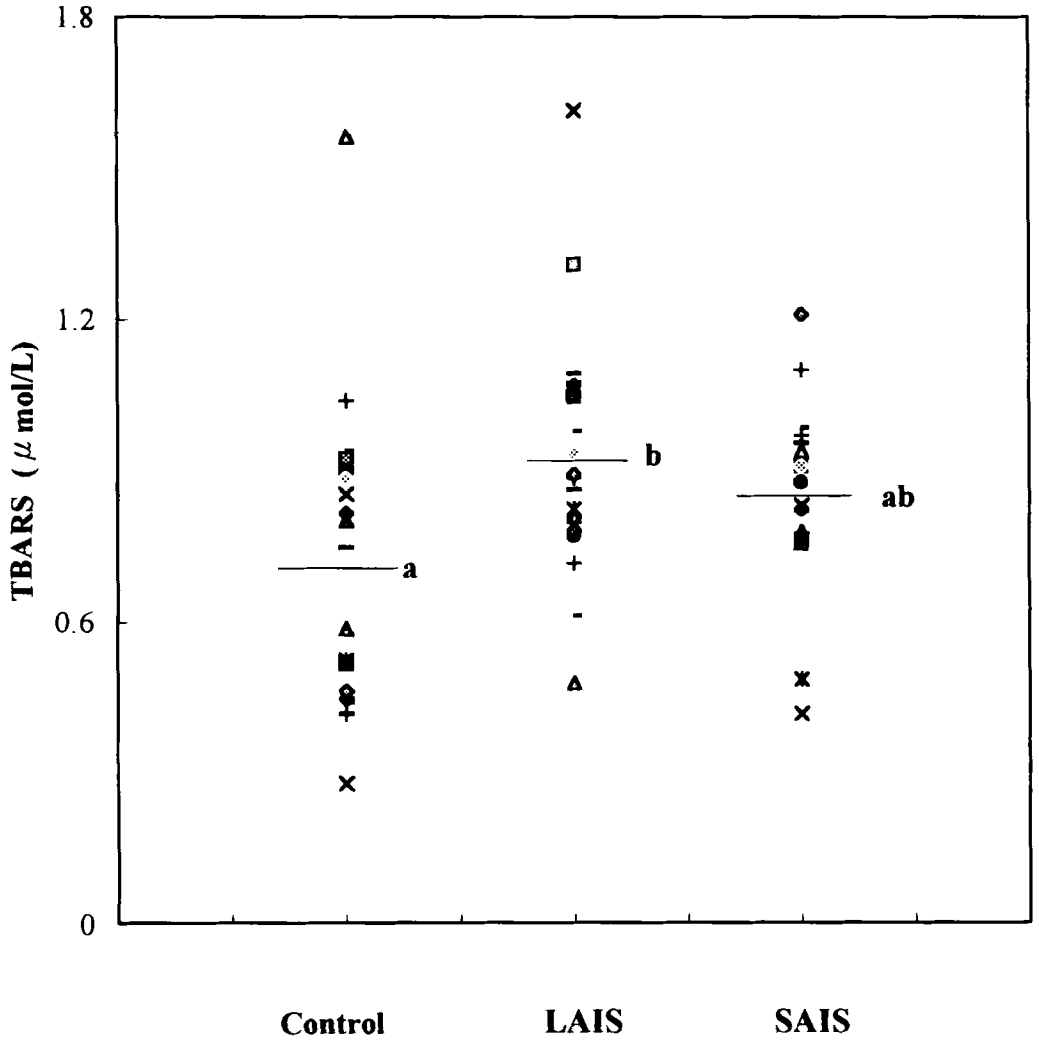

FIGURE 4 Distribution of plasma levels of TBARS in the controls (n = 21) and patients with large-artery ischemic stroke (LAIS, n = 19) and small-artery ischemic stroke (SAIS, n = 17). Symbols and bars are the same as those described in Figure 1.

0.6 μM for the patients. Results from the present study showed that the plasma levels of TBARS of our patients (0.89 ± 0.23 μM) and matched controls (0.71 ± 0.30 μM) are comparable to those reported using the HPLC-based assay. This is likely due to the TCA-precipitating step which removes most[26] (but not all) of interfering materials (see the Methods) before the acid-heating step during the TBA assay. Thus, the elevated level of plasma TBARS in our ischemic-stroke patients is suggestive of an enhanced lipid peroxidation soon after the onset of ischemic stroke. This is consistent with the findings in animal studies.[10–12,40,41] For instance, Tomita *et al.*[40] have shown that the levels of TBARS in the blood from the stroke-prone spontaneously hypertensive (SHRSP) rats with cerebral lesions are more than doubled as compare to those of the healthy SHRSP rats. However, there was no evidence of protein damage in our patients since protein carbonyls, a marker of oxidative protein damage produced oxidatively from amino acid side

chains and/or the peptide backbone,[42,43] and T-SH groups (consisting largely of thiol groups from albumin that are susceptible to oxidative damage),[26,44] remained unchanged within 24 hrs after the onset of ischemic stroke. The discrepancy in oxidative damage to lipids and proteins means that the origin of elevated plasma TBARS in our patients is uncertain. It may be that the patients had preformed plasma TBARS or lipid peroxides, either by enzymatic and non-enzymatic reactions or by intakes from dietary fats containing hydroperoxides, which may play a notable role in the provocation of ischemic stroke.[37] Chirico *et al.*[39] have suggested that atherosclerosis leads to elevated plasma TBARS by escape of oxidized low-density lipoproteins from atherosclerotic lesions.

Higher carotenoids may be important in protection against stroke, although it is unclear whether this is related to their radical scavenging (including singlet oxygen quenching) ability.[45,46] Nevertheless, plasma carotenoids may at least be a marker of vegetable and fruit consumption which is a risk factor of stroke.[13–16] We have no reference value of plasma carotenoids in people of Taiwan, but dietary surveys[35,36] have demonstrated that approximately 75% of food sources for vitamin A are of plant origin.

The separation of our patients into LAIS and SAIS groups revealed slight differences in antioxidant and oxidative status, namely, vitamin A level was only significantly lower and TBARS were only significantly higher in the LAIS group than the controls. The significance of such findings is not clear at present because of the relatively few patients in both LAIS and SAIS groups. Overall, our study demonstrates that ischemic-stroke patients have lower levels of fat-soluble antioxidants and higher levels of lipid peroxidation products than the matched controls within 24 hrs after the onset of the clinical event. Although the results suggest that lowered antioxidants may be a result of enhanced lipid peroxidation in ischemic stroke, the opposite may also be true since our study does not exclude the possibility that lipid peroxidation may have occurred before the clinical event.

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References

[1] (1994). Heart and stroke facts: 1994 Statistical supplement, American Heart Association, Dallas, TX.
[2] Archives of Public Health, (1994). Department of Public Health, Executive Yuan, R.O.C.
[3] Halliwell, B. and Gutteridge, J. M. C. (1989). *Free Radicals in Biology and Medicine*, 2nd ed., (Clarendon Press, Oxford).
[4] Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M. and Harman, D. (1987). Oxygen radicals and human disease. *Annals of Internal Medicine*, **107**, 526–545.
[5] Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative disease of aging. *Proceedings of Natational Academy of Science USA*, **90**, 7915–7922.
[6] Cutler, R. G. (1991). Antioxidants and aging. *American Journal of Clinical Nutrition*, **53**, 373s–379s.
[7] Yoshida, S., Inoh, S., Asano, T., Sano, K., Kubota, M., Shimasaki, M. and Ueta, N. (1980). Effect of transient ischemia on free fatty acids and phospholipids in the gerbil brain: lipid peroxidation as possible cause of postischemic injury. *Journal of Neurosurgery*, **53**, 323–331.
[8] Kogure, K., Watson, B. D., Busto, R. and Abe, K. (1982). Potentiation by lipid peroxides by ischemia in rat brain. *Neurochemical Research*, **7**, 437–454.
[9] Yamamoto, M., Shima, T., Uozumi, T., Sogabe, T., Yamada, Y. and Kawasaki, T. (1983). A possible role lipid peroxidation in cellular damages caused by cerebral ischemia and the protective effect of α-tocopherol administration. *Stroke*, **14**, 977–982.
[10] Watson, B. D., Busto, R., Goldberg, W. J., Santiso, M., Yoshida, S. and Ginsberg, M. D. (1984). Lipid peroxidation in vivo induced by reversible global ischemia in rat brain. *Journal of Neurochemistry*, **42**, 268–274.
[11] Imaizumi, S., Kayama, T. and Suzuki, J. (1984). Correlation between energy metabolism and free radical reaction. *Stroke*, **15**, 1061–1065.
[12] Yoshida, S., Abe, K., Busto, R., Watson, B. D., Kugure, K. and Ginsberg, D. (1982). Influence of transient ischemia on lipid-soluble antioxidants and free fatty acids and energy metabolism in brain. *Brain Research*, **245**, 307–316.

[13] Acheson, R. M. and Williams, D. R. R. (19?8). Does consumption of fruit and vegetables protect against stroke? *Lancet*, **1**, 1191–1193.
[14] Manson, J. E., Willet, W. C., Stampfer, M. J., Colditz, G. A., Speizer, F. E. and Hennekens, C. H. (1994). Vegetable and fruit consumption and incidence of stroke in women. *Circulation*, **89**, 932 (Abstract).
[15] Gillman, M. W., Cupples, L. A., Gagnon, D., Posner, B. M., Ellison, R. C., Castelli, W. P. and Wolf, P. A. (1995). Protective effect of fruits and vegetables on development of stroke in men. *Journal of American Medical Association*, **273**, 1113–1117.
[16] Manson, J. E., Stampfer, M. J., Willet, W. C., Colditz, G. A., Speizer, F. E. and Hennekens, C. H. (1993). Antioxidant vitamin consumption and incidence of stroke in women. *Circulation*, **87**, 678 (Abstract).
[17] Blot, W. J., Li, J. Y., Taylor, P. R., Guo, W., Dawsey, S. M. and Li, B. (1995). The Linxian trials: mortality rates by vitamin-mineral intervention group. *American Journal of Clinical Nutrition*, **62** (suppl), 1424S–1426S.
[18] Gey, K. F., Stahelin, H. B. and Eichholzer, M. (1990). Poor plasma status of carotene and vitamin C is associated with higher mortality from ischemic heart disease and stroke: Basel Prospective Studies. *Clinical Investigation*, **71**, 3–6.
[19] Gey, K. F., Morter, U. K., Jordan, P., Stahelin, H. B., Eichholzer, M. and Ludin, E. (1990). Increased risk of cardiovascular disease at suboptimal plasma concentrations of antioxidants: an epidemiological update with special attention to carotene and vitamin C. *American Journal of Clinical Nutrition*, **57**, 787s–797s.
[20] Kok, F. J., DeBruijin, A. M., Vermeeren, R., Hofman, A., VanLaar, A., DeBruin, M., Hermus, R. J. J. and Valkenberg, H. A. (1987). Serum selenium, vitamin antioxidants and cardiovascular mortality: a 9-year follow-up study in the Netherlands. *American Journal of Clinical Nutrition*, **45**, 462–468.
[21] Driskell, W. J., Lackey, A. D., Hewett, J. S. and Bashor, M. M. (1985). Vitamin A stability in frozen sera. *Clinical Chemistry*, **31**, 871–872.
[22] Gunter, E. W., Driskell, W. J. and Yeager, P. R. (1988). Stability of vitamin E in long-term stored serum. *Clinical Chemical Acta*, **175**, 329–336.
[23] Nino, H. V. and Shaw, W. (1982). *The Vitamins in Fundamentals of Clinical Chemistry* N. W. Tietz (ed.), (Saunders, Philadelphia), pp. 542–547.
[24] Hu, M.-L. (1994). Measurement of protein thiol groups and glutathione in plasma. *Methods in Enzymology*, **233**, 380–385.
[25] Levine, R. L. (1983). Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *Journal of Biological Chemistry*, **258**, 11828–11833.
[26] Rodriguez, M. A. and Ruiz-Torres, A. (1992). Homeostasis between lipid peroxidation and antioxidant enzyme activities in healthy human aging. *Mechanism Ageing Development*, **66**, 213–222.
[27] Koh, T. S. and Benson, T. H. (1983). Critical re-appraisal of fluorometric method for determination of selenium in biological materials. *Journal of Association of Official Analytical Chemistry*, **66**, 918–926.
[28] Chen Huang, L., Hsu, S. J., Huang, P. C. and Chen, J. S. (1977). Vitamin E status of Chinese population in Taiwan. *American Journal of Clinical Nutrition*, **30**, 728–735.
[29] Goode, H. F., Cowley, H. C., Walker, B. E., Howdle, P. D. and Webster, N. R. (1995). Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. *Critical Care Medicine*, **23**, 646–651.
[30] Fujimoto, S., Mizoi, K., Yoshimoto, T. and Suzuki, J. (1984). The protective effect of vitamin E on cerebral ischemia. *Surgical Neurology*, **22**, 449–454.
[31] Hara, H., Kato, H. and Kogure, K. (1990). Protective effect of α-tocopherol on ischemic neuronal damage in the gerbil hippocampus. *Brain Research*, **310**, 333–338.
[32] Suzuki, J., Fujimoto, S., Mizor, K. and Oba, M. (1984). The protective effect of combined administration of antioxidants and perfluorochemicals on cerebral ischemia. *Stroke*, **15**, 672–679.
[33] Ames, B. N., Cathcart, R., Schwiers, E. and Hochstein, P. (1981). Uric acid provides an antioxidant defense in humans against oxidant and radical-caused aging and cancer: a hypothesis. *Proceedings of National Academy of Science USA*, **78**, 6858–6863.
[34] Gey, K. F. (1995). Ten-year retrospective on the antioxidant hypothesis of arteriosclerosis: threshold plasma levels of antioxidant micronutrients related to minimum cardiovascular risk. *Journal of Nutritional Biochemistry*, **6**, 206–236.
[35] Lee, N. Y., Chu, Y. C., Chang, C. P., Shieh, M. J. and Kao, M. D. (1991). Dietary survey in Taiwan area, 1986–1988. *Journal of the Chinese Nutrition Society*, **16**, 39–60.
[36] Pan, W. H., You, S. L., Hsu, C. P., Chou, J. and Huang, P. C. (1991). Major food contributors of various nutrients consumed by Chinese populations in Taiwan, 1980–1981, (II): vitamins and minerals. *Journal of the Chinese Nutrition Society*, **16**, 21–37.
[37] Yagi, K. (1987). Lipid peroxidation and Human disease. *Chemistry and Physics of Lipids*, **45**, 337–351.
[38] Wade, C. R. and van Rij, A. M. (1989). Plasma malonaldehyde, lipid peroxides, and the thiobarbituric acid test. *Clinical chemistry*, **35**, 336–342.
[39] Chirico, S., Smith, C., Marchant, C., Mitchinson, M. J. and Halliwell, B. (1993). Lipid peroxidation in hyperlipidemic patients. A study of plasma using an HPLC-based thiobarbituric acid test. *Free Radical Research Communications*, **19**, 51–57.
[40] Tomita, I., Sano, M., Serizawa, S., Takata, T., Furukawa, M. and Ohta, K. (1979). Fluctuation of lipid peroxides and related enzyme activities at time of stroke in stroke-prone spontaneous hypertensive rats. *Stroke*, **10**, 323–326.
[41] Tomita, I., Sano, M., Serizawa, S., Takata, T., Furukawa, M. and Ohta, K. (1979). Fluctuation of lipid peroxides and related enzyme activities during the development of stroke in stroke-prone spontaneous hypertensive rats. *Japanese Heart Journal*, **20** (suppl 1), 328–330.
[42] Oliver, C. N., Ahn, B. W., Moerman, E. J., Goldstein, S. and Stadtman, E. R. (1987). Age-related changes in oxidized proteins. *Journal of Biological Chemistry*, **262**, 5488–5491.
[43] Stadtman, E. R. (1992). Protein oxidation and aging. *Science*, **257**, 1220–1224.
[44] Halliwell, B. (1988). Albumin—an important extracellular antioxidant? *Biochemical Pharmacology*, **37**, 569–571.
[45] Foote, C. S. and Denny, R. W. (1968). Chemistry of singlet oxygen. VIII. Quenching by β-carotene. *Journal of American Chemical Society*, **90**, 6233–6235.
[46] Krinsky, N. I. and Deneke, S. M. (1982). The interaction of oxygen and oxygen radicals. *Journal of National Cancer Institute*, **69**, 205–210.