Small Molecule Copper and Its Relative Metabolites in Serum of Cerebral Ischemic Stroke Patients

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Background: Copper is a strong pro-oxidant. The most important pro-oxidative form in serum is small molecule copper (SMC), which is copper that is loosely bound to small molecules, such as amino acids and polypeptides. The association between copper and atherosclerotic diseases has been confirmed, but that between SMC and cerebral ischemic stroke (CIS), one of the most principal manifestations and causes of death of atherosclerotic disease, is not yet clear. *Methods:* We recruited 45 CIS patients and 25 age- and gender-matched healthy controls. We detected their serum levels of SMC, total copper, homocysteine (Hcy), and ceruloplasmin (CP), as well as urinary total copper, and analyzed the relationship of SMC with these aforementioned metabolites or compounds in CIS patients. *Results:* SMC was 4.2 ± .5 μg/L and 2.1 ± .9 μg/L; total copper in sera was 1345.5 ± 308.2 μg/L and 1180.3 ± 134.0 μg/L; and total copper in urine was 27.6 ± 9.3 μg/L and 18.8 ± 8.1 μg/L in patients and controls, respectively (all $P < .05$). Serum CP activity in CIS patients was 59.92 ± 12.11 U/L versus 37.76 ± 5.71 U/L in controls ($P = .0001$). The concentration of SMC was positively correlated with CP activity, Hcy concentration in sera, and urinary total copper. *Conclusion:* The serum level of SMC and total copper is remarkably elevated, and SMC positively correlates with Hcy, CP activity, and urinary total copper in CIS patients. **Key Words:** Small molecule copper—cerebral ischemic stroke—ceruloplasmin—homocysteine.

Introduction

Copper is an essential trace element in humans. It has several roles, including the synthesis of phospholipids, formation of hemoglobin, and transport of electrons. About 95% of the copper in plasma is bound firmly in a protein complex, ceruloplasmin (CP), or in relevant enzymes, such as Cu/Zn superoxide dismutase and cytochrome C oxidase.[1] The remaining 5% is loosely bound to small molecules, such as amino acids and polypeptides, and this kind of copper is defined as small molecule copper (SMC) in our current study. SMC, being previously considered as "free" copper in serum, is harmful to human health due to its strong oxidative effect.[2] It can cause oxidative modification of low-density lipoprotein cholesterol (LDL-c) and the formation of free radicals, participating in atherogenesis.[3,4]

The association between serum copper concentration and atherosclerotic disease has been confirmed in previous studies. For example, in the Ludwigshafen Risk and Cardiovascular Health Study, Grammer et al[5] analyzed 3253 participants and found that serum copper and CP concentrations are positively associated with angiographic coronary atherosclerotic disease and mortality. Bagheri's

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et al[6] similarly found that serum copper is elevated and increases with the severity of atherosclerosis in atherosclerotic patients. Ford[7] found that the age-adjusted serum copper concentration was approximately 5% higher among 4574 participants who died from coronary atherosclerotic disease than among those who did not. Iskra et al[8] found that femoral atherosclerosis patients have elevated serum copper concentrations, which is strongly positively correlated with total cholesterol and LDL-c ($r > .89$). A relationship between plasma copper and ischemic stroke has also been suggested. A metallomics-based study showed that plasma copper concentration in ischemic stroke patients is significantly higher than those of hemorrhagic stroke and migraine controls.[9] Altamura et al[10] discovered a significantly higher content of total copper and "free" copper in the serum of cerebral ischemic stroke (CIS) patients. However, it is still not clear whether it is SMC or total copper in the serum that is related to ischemic stroke. This question is important because it is SMC, but not total copper, in serum that really exerts an oxidative effect. Thus, we aim to investigate the change in SMC, as well as the constituents or metabolites that affect or are correlated with SMC, including serum total copper, CP, homocysteine (Hcy), and urinary total copper in CIS patients, and to explore the change law of SMC and its influencing factors in CIS patients.

Methods

Subjects and Sample Collection

The study was approved by the ethics committee of Shantou University Medical College, and written informed consent was obtained from all subjects. Forty-five patients diagnosed with acute CIS, as well as 25 age- and gender-matched healthy controls, were enrolled from the First Affiliated Hospital of Shantou University Medical College. Acute CIS was confirmed by magnetic resonance imaging or computed tomography and clinical characteristics of patients according to the recommendations by the American Heart Association/American Stroke Association.[11] Of these 45 patients, 10 had undergone computed tomography, and 35 had undergone magnetic resonance imaging. All had lesions that could be attributed to cerebral artery occlusion. Among these occlusions, 27 cases were anterior circulation infarction, of which 5 were bilateral anterior circulation, 14 cases were posterior circulation infarction, and 4 cases had both anterior and posterior infarctions. All participants were recruited within 7 days of symptom onset. Exclusion criteria were (1) the presence of illnesses, including renal and liver disease, malignancy, autoimmune disease, and hypothyroidism, and (2) the inability to comply with a magnetic resonance examination.

Blood samples were obtained by venipuncture after fasting overnight and collected in glass tubes containing coagulant. After 10 minutes at room temperature, the serum was separated by centrifugation at 3000 rpm for 10 minutes at 4°C. The first morning urines were collected and centrifuged at 5000 rpm for 10 minutes to obtain approximately 2 mL of supernatant. The serum and urine samples were stored at –80°C until analysis. The detailed clinical characteristics of patients are described in Table 1.

Table 1. *Clinical characteristics of subjects*

Characteristics	Ischemic stroke patients (n = 45)	Healthy controls (n = 25)	*P*
Gender (M/F)	25/20	15/10	.458
Age (years)	61.2 (31-77)	58.0 (53-66)	.100
Diabetes	18	3	.016
Hypertension	37	7	<.001
Hcy (μmol/L)	17.86	13.38	.012
LDL-c (mmol/L)	4.03	3.51	.041
HSCRP (mg/L)	9.10	3.57	.038

Abbreviations: F, female; Hcy, homocysteine; HSCRP, human serum C-reactive protein; LDL-c, low-density lipoprotein cholesterol; M, male.

Detection of Total Copper in Serum and Urine, and SMC in Serum

Total copper in serum and urine was measured by graphite furnace atomic absorption spectrometry after the samples had been diluted 50 and 5 times, respectively, with .5% hydrogen nitrate (HNO_3), according to our previous study.[12]

Ultrasonic Oscillation Dialysis Procedures for Assessment of SMC

Each dialysis device for convenient, small-volume (.2 mL) sample dialysis was made in-house using a 1 mL sample cell and a .5-mL Eppendorf tube whose tube end had been cut and replaced with a 25 kD dialysis membrane. Serum (200 μL) was added to the inner tube, and 500 μL of phosphate-buffered saline (100 mmol/L) was added to the outer tube. Then, the dialysis device was placed in an ultrasonic cleaner (Automatic Science Instrument Co., Ltd., Oakville, Ontario, Canada) and ultrasonically oscillated for 30 minutes. Then, the dialysate in the outer tubes was collected for copper determination by graphite furnace atomic absorption spectrometry.[12]

Determination of CP and Hcy Concentrations, and CP Activity in Sera

CP concentrations in sera were determined by scattering immunoturbidimetric assay. CP activities in sera were determined by colorimetry, using a CP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Hcy was detected by an enzymatic method in an automatic