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BLOOD TRACE MINERALS CONCENTRATIONS AND OXIDATIVE STRESS IN PATIENTS WITH OBSTRUCTIVE SLEEP APNEA

P.-C. CHEN[1], C.-H. GUO[1], C.-J. TSENG[2], K.-C. WANG[3], P.-J. LIU[4]

1. Biomedical Nutrition & Micro-Nutrition Labs, Institute of Biomedical Nutrition, Hung Kuang University, Taichung 433, Taiwan, Republic of China; 2. Nutrition Room, Yuan-Rung General Hospital, Changhua 510, Taiwan, Republic of China; 3. Department of Otolaryngology, Cheng-Ching Hospital, Taiwan Boulevard, Taichung 407, Taiwan, Republic of China; 4. Department of Otolaryngology & Nutrition and Naturopathic center, Cheng-Ching Hospital, Taiwan Boulevard, Taichung 407, Taiwan, Republic of China ; PhD student, School of Nutrition, Chung Shan Medical University, Taichung 402, Taiwan, Republic of China. Corresponding author: Po-Jen Liu, E-mail:xz48899959@gmail.com
Tel: 886-4-2463-2000 ext. 53664, Fax: 886-4-2463-5961

Abstract: *Background:* Obstructive sleep apnea (OSA) is associated with increased oxidative stress. Certain essential trace minerals have shown to play an important role in the maintenance of redox homeostasis. We determined the concentrations of trace minerals in OSA patients and assessed their relationships to OSA severity as indicated by the apnea/ hypopnea index (AHI). *Methods:* We enrolled 44 patients with newly diagnosed mild to moderate OSA and 20 without OSA. The following parameters were measured: polysomnographic values of nocturnal sleep; plasma trace minerals zinc (Zn), copper (Cu), iron (Fe), and erythrocyte selenium (Se); oxidative stress status; and plasma high-sensitivity C-reactive protein (hs-CRP) and tumor necrosis factor-α (TNF-α). *Results:* Compared to controls matched for age, gender, and body mass index, OSA patients had lower concentrations of plasma Zn and erythrocyte Se and higher plasma concentrations of Cu and Fe. OSA patients had significantly higher plasma concentrations of hs-CRP, TNF-α, and malondialdehyde (MDA), and lower erythrocyte antioxidant enzyme glutathione peroxidase (GPx) and superoxide dismutase activities. Significant differences in all the above parameters were also found in patients with moderate OSA compared to those with mild OSA. Furthermore, AHI values correlated significantly with neck circumference, GPx activity, and MDA, hs-CRP, and TNF-α concentrations in OSA patients. AHI values were also negatively associated with concentrations of plasma Zn and erythrocyte Se, but were positively linked to plasma concentrations of Fe and Cu. *Conclusions:* Abnormal concentrations of these trace minerals may reflect oxidative damage and inflammatory response, thus increasing the severity of OSA.

Key words: Trace minerals, oxidative stress, inflammation, obstructive sleep apnea.

Introduction

Obstructive sleep apnea (OSA) is a major public health problem of increasing concern worldwide. This common sleep-related breathing disorder is characterized by repeated episodes of upper airway collapse, resulting in multiple periods of hypoxia and re-oxygenation (1). In addition, these repeated episodes of transient cessation of breathing can increase oxidative stress and inflammation in OSA patients (2). It has been shown that OSA is associated with increased cardiovascular morbidity, independent of obesity and percentage of body fat (3). OSA patients may require long-term nightly use of nasal continuous positive airway pressure, while this treatment only partial reverses oxidative stress and arterial dysfunction (4).

Recent evidence suggests that oxidative damage plays a deleterious role in progressive OSA and cardiovascular disease (5). Oxidative stress, defined as a disruption in the balance between pro-oxidant and antioxidant systems, can lead to oxidative damage. Oxidative stress in patients with OSA can induce propagation of inflammatory cascades that can cause atherogenesis and vascular dysfunction (6). Those suffering from moderate to severe OSA have been found to have significantly higher oxidative stress and lower total antioxidant capacity (4, 7, 8). Compared to healthy subjects, patients with severe OSA were found to have decreased glutathione status and altered activities of blood antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (9, 10). Thus, OSA may be associated with increased oxidative stress via glutathione redox potential (8, 9).

On the other hand, essential trace minerals such as zinc (Zn), copper (Cu), selenium (Se), and iron (Fe) are known to play important roles in the maintenance of redox homeostasis. Alterations in the status of these minerals may result in stronger inflammatory responses and increased oxidative stress (11). Zn is critical to the structure of SOD and can stabilize biological membranes to decrease their susceptibility to oxidative damage. Cu is required for the catalytic activity of SOD, and Cu deficiency or excess can induce oxidative stress that can lead to chronic inflammation and affect immune responses. Fe is a constituent of the enzyme catalase. Free Fe can participate in the Fenton reaction, catalyzing the generation of hydroxyl radicals. Se bound to the active site of GPx plays an important role in protecting cell membranes from oxidative damage. Se also attenuates inflammatory responses and is required for immune system function in disease conditions (12). Alterations in the immune system have been shown to play an important role in the development of OSA complications (13, 14). This observation suggests that OSA may create disturbances in the homeostasis of these trace minerals, leading to oxidant-antioxidant imbalance and thus increasing the deleterious effects of OSA. However, information regarding the

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concentrations of plasma trace minerals in OSA patients is limited.

In the present preliminary study, concentrations of selected trace minerals in plasma or erythrocyte, erythrocyte antioxidant enzyme activities, and inflammatory status (as assessed by levels of high-sensitivity C-reactive protein [hs-CRP] and tumor necrosis factor-α [TNF-α]), and lipid peroxidation products were determined in patients with recently diagnosed mild to moderate OSA. The relationships of the severity of OSA to trace minerals and oxidative stress were also examined.

Methods

Subjects

From April 2010 to April 2011, 64 consecutive participants at the sleep disorders medical center of Cheng-Ching Hospital (Taichung, Taiwan) were enrolled. None of the subjects was a smoker or alcohol consumer. Subjects with hypertension, cardiovascular diseases, dyslipidemia, diabetes mellitus, narcolepsy, chronic obstructive pulmonary disease, thyroid dysfunction, systemic inflammatory disorders, and taking any type of medication or supplementation with antioxidants were excluded. Patients who had severe OSA or currently using nasal continuous positive airways pressure therapy were also excluded. All of the subjects signed consent statements and agreed to participate in the study. The study protocol was approved by the ethics in human research committee of Cheng-Ching Hospital (IRB, #HP090024, HP120002).

Demographic information, objective measurement for excessive daytime sleepiness (Epworth Sleepiness Scale, ESS) (15), and anthropometric measurements such as height, weight, body mass index (BMI), neck and waist circumference, and blood pressure were obtained before polysomnography (PSG). All subjects underwent overnight PSG and the diagnosis of OSA was measured using full PSG (Alice 4 Diagnostics System; Healthdyne, Atlanta, GA). PSG consisted of continuous recording of EEG, electrooculography, electromyography, ECG, thoracic and abdominal impedance belts for respiratory effort, thermistors for nasal and oral airflow, pulse oximetry, and tracheal microphone for snoring. Sleep was scored every 30-second epoch of the nocturnal PSG based on the Rechtschaffen-Kales criteria (16). The oxygen saturation level (SpO2) was also calculated transcutaneously with fingertip pulse oximetry.

Apnea/hypopnea index (AHI) was defined as the total number of apneas and hypopneas per hour of sleep, and oxygen desaturation index (ODI) was calculated as the number of oxygen desaturations (≥4%) per hour of sleep. Apnea was defined as complete cessation of airflow for at least 10 seconds. Hypopnea was defined as a substantial reduction in airflow (>50%) for at least 10 seconds or a moderate reduction in airflow for at least 10 seconds associated with electro-encephalographic arousals or oxygen desaturation (≥4%). All participants were categorized into different groups by OSA severity. Subjects with AHI less than 5 events/hour (/h) were grouped as normal control; AHI of ≥5/h to <15/h, and <30/h were classified as mild OSA and moderate OSA, respectively; subjects with AHI ≥30/h were classified as severe OSA. Subjects had severe OSA were excluded from this study.

Biochemical Analysis

On the morning after PSG, between 7:00-8:00, peripheral venous blood samples were collected in BD-Vacutainer tubes (sodium heparin) after an overnight fast of at least 8 h. Plasma concentrations of hemoglobin, total cholesterol, triglyceride, and glucose were estimated using routine laboratory techniques at hospital Department of Laboratory Medicine.

Measurement of Oxidative Stress and Inflammation

The extent of lipid peroxidation was determined by assaying the formation of malondialdehyde (MDA). Plasma samples were mixed with 3% sodium dodecyl sulfate, 0.1 N HCl, 10% phosphotungstic acid, and thiobarbituric acid, and then incubated at 95°C for 60 min. The n-butanol was added and the mixture was shaken vigorously. After centrifugation at 12,000 x g and 4 °C for 15 min, the absorbance of the upper layer was read at 530 nm with excitation at 485 nm (17).

Plasma concentrations of hs-CRP were measured using the human CRP ELISA kit (E-80CRP, Immunology Consultants Laboratory, Inc, Newberg, OR, USA). The intra- and inter-assay CVs (coefficient of variation) were < 3% and < 4%, respectively. Briefly, 200 µl of combined enzyme-antibody conjugate and TMB substrate was added to each well and was incubated in darkness at room temperature. After 10 min the reaction was stopped with the addition of stop solution. The absorbance at 450 nm was measured, and readings were interpolated into the standard curve. In addition, plasma concentrations of TNF-α (#88-7346, human ELISA Ready-SET-Go, eBioscience, San Diego, CA, USA) were measured. Briefly, plasma samples were added to the wells and incubated with anti-TNF-α antibodies for overnight. Combined avidin-HRP and substrate solution was added to each well and was incubated at room temperature. Absorbance at 450 nm was measured, and readings were interpolated into the standard curve.

Determination of Trace Minerals

The concentrations of plasma Zn, Cu, and Fe were measured with a flame atomic absorption spectrophotometer (932 plus, GBC, Australia) using an air-acetylene flame without background correction at 213.9, 324.71, and 278.8 nm, respectively. Triplicate absorbance readings were taken for each sample in the peak-height mode. Samples were digested in a H2O2/HNO3 mixture in a START D microwave-assisted digestion system (Milestone Microwave Labstation ETHOSD), and the volume was increased with double deionized water.

Further, the accessory hydride generation system (HG 3000), from GBC, was used for determining erythrocyte Se

concentrations. Hollow cathode lamps were employed at the 196.0 nm wavelength and 1.0 nm band pass. The temperature programmed for the atomization was based on recommendation of the manufacturer. Samples were digested for a total of 10.5 h with initial temperature of 60°C for 1-1.5 h, followed by increasing temperatures on 20°C increments and finally heated up to 225°C for 2 h in a mixture of 3.2 mL nitric acid (16N), and 0.8 mL concentrated perchloric acid to convert all Se species to selenate. The reduction of selenate was completed within 30 min at a block temperature of 120°C (18, 19). Evaluation of the accuracy of the methods was confirmed by comparing to serum (level 2, NO0371) reference materials (Seronorm, Nycomed, Oslo, Norway). The values of intra-assay CV% for Zn, Cu, Fe, and Se were 3.3%, 4.5%, 2.7%, and 3.1%, respectively; the CV% inter-assay were 3.9%, 5.0%, 2.9%, and 3.7%, respectively.

Measurement of Enzyme Activities

The erythrocyte pellets were washed three times with cold isotonic saline. Lysate of erythrocytes was prepared by adding distilled water and keeping the mixture at 4°C for 15 min. Erythrocyte SOD activity was determined with a SOD assay kits (Cayman, Ann Arbor, Michigan, USA); one unit was defined as the amount of enzyme necessary to produce 50% dismutation of the superoxide radicals. The activity of SOD was expressed in unit per gram of hemoglobin (U/mg Hb). In addition, GPx activity measured with a kit from Cayman Chemical (cat #703102); the rate of decrease in absorbance at 340 nm is directly proportional to the GPx activity.

Statistical analysis

Quantitative variables were expressed as mean (SD) or median (IQR). A two-tailed p value less than 0.05 was considered statistically significant. The Shapiro-Wilk test was applied to evaluate the distribution of variances. Comparisons of different variables were made by one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA, as appropriate. In addition, Pearson's or Spearman's correlation coefficients were performed to identify correlations of blood variables.

Results

Clinical characteristics

No significant differences in age, sex, BMI, waist circumference, and WHR were found among patients with mild, moderate, and no OSA ($p > 0.05$) (Table 1). The neck circumference for patients with moderate OSA was higher than for mild OSA and no OSA. Compared to controls, there was no significant difference in EES scores and SpO2 values in OSA patients. These patients showed higher ODI values, as compared to that were observed in the patients with no OSA. Further, the values of moderate OSA patients were higher than those found in the mild OSA patients. There was no significant difference in plasma concentrations of hemoglobin, triglyceride, and glucose between OSA patients and individuals without OSA; whereas patients with moderate OSA syndrome had higher plasma concentrations of total cholesterol.

Table 1
Baseline characteristics of the study group subjects[1,2,3]

	no OSA (n=20)	mild OSA (n=23)	moderate OSA (n=21)
Age (yrs)	42 ± 11	40 ± 11	45 ± 13
Gender (M/F)	15/5	17/6	16/5
Height (cm)	167 ±6	168 ± 6	171 ± 6
Weight (kg)	71 ± 7	77 ± 8	79 ± 9
Body mass index, BMI (kg/m^2)	26.0 ± 3.3	27.5 ± 4.2	26.7 ± 2.8
Neck circumference (cm)	34.8 ± 3.1 [a]	36.6 ± 3.5 [a]	39.0 ± 2.9 [b]
Waist circumference (cm)	84.1 ± 9.5	90.7 ± 11.1	89.7 ± 8.7
Waist-to-hip ratio, WHR	0.85 ± 0.08	0.87 ± 0.06	0.88 ± 0.08
Systolic blood pressure (mmHg)	117 ± 11	127 ± 17	127 ± 12
Diastolic blood pressure (mmHg)	71 ± 11	75 ± 11	77 ± 7
ESS	7.0 ± 2.0	7.0 ± 3.7	7.3 ± 2.9
Apnea-hypopnea index, AHI (/h)	3.3 ± 0.9 [a]	8.6 ± 2.9 [b]	21.1 ± 2.8 [c]
Oxygen desaturation index, ODI (/h)	1.0 ± 0.8 [a]	5.6 ± 3.6 [b]	15.0 ± 4.9 [c]
SpO2 (%)	95 (89-96)	95 (87-96)	93 (83-94)
Blood test			
Hemoglobin (gm%)	14.5 ± 1.4	14.4 ± 1.4	15.1 ± 1.4
hs-CRP (ug/mL)	0.1 (0.0-0.2) [a]	0.3 (0.1-0.9) [b]	0.7 (0.2-1.3) [c]
TNF-α, (pg/mL)	1.2 (0.5-1.7) [a]	2.8 (0.8-4.1) [b]	3.8 (2.5-9.9) [c]
MDA (nmol/L)	4 (3.2-4.2) [a]	5.2 (4.0-5.5) [b]	5.9 (4.9-7.4) [c]
Total cholesterol (mg/dL)	170 ± 32 [a]	197 ± 30 [a]	215 ± 33 b
Triglyceride (mg/dL)	137 (94-170)	157 (113-169)	156 (129-181)
Blood sugar (mmol/L)	4.9 (4.4-5.3)	5.3 (4.9-5.6)	5.5 (5.0-6.0)
SOD (U/mg Hb)	3.7 (3.0-4.1) [c]	2.4 (1.6-3.7) [b]	1.9 (1.7-2.9) [a]
GPx (nmol/min/mg Hb)	13.4 (11.9-14.2) [c]	11.9 (8.4-12.5) [b]	10.0 (7.8-10.9) [a]

1. Values are mean ± SD or medians (inter-quartile range). 2. Values in the same row with different superscripts are significantly different ($p < 0.05$). 3. ESS = Epworth Sleepiness Scale; SpO2 = oxygen saturation; hs-CRP = high-sensitivity C-reactive protein; TNF-α = tumor necrosis factor-α; MDA = malondialdehyde; SOD = superoxide dismutase; GPx = glutathione peroxide.

Oxidative stress, antioxidant enzymes, and trace mineral levels

The concentrations of plasma markers of oxidative stress, MDA, were considerably increased in OSA patients compared to the controls. We found higher plasma hs-CRP and TNF-α concentrations in these patients (Table 1). In addition, patients with moderate OSA had maximal concentrations of MDA, hs-CRP, and TNF-α, and minimal erythrocyte antioxidant enzyme activities of SOD and GPx.

Compared to the controlled subjects, patients with mild OSA had non-significantly lower erythrocyte Se concentrations. On contrary, the concentrations of Zn and Se were reduced in patients with moderate OSA. Markedly higher plasma concentrations of Cu and Fe were observed in OSA patients, compared with those found in the controlled subjects (Fig. 1).

Correlations between AHI and other parameters in OSA patients

For OSA patients, AHI values showed statistically significant relationships to plasma MDA ($r = 0.504$), hs-CRP ($r = 0.398$), TNF-α ($r = 0.479$) concentrations, and antioxidant enzyme GPx activity ($r = -0.384$). AHI values were also

significantly related to neck circumference (r = 0.412)(Fig. 2). Significant correlations between AHI values and plasma Zn (r = -0.484), Cu (r = 0.515), Fe (r = 0.473), and erythrocyte Se (r = -0.392) concentrations were obtained (Fig. 3). Additionally, plasma concentration of Fe was associated with plasma TNF-α concentration (r = 0.391, p = 0.009) (data not shown).

Figure 1

The plasma concentrations of trace minerals (a)Zn, (b)Cu, (c)Fe, and (d) erythrocyte Se.
Values are mean ± SD or medians (inter-quartile range). Values with different superscripts are significantly different ($p < 0.05$).
Zn = zinc; Cu = copper; Fe = iron; Se = selenium

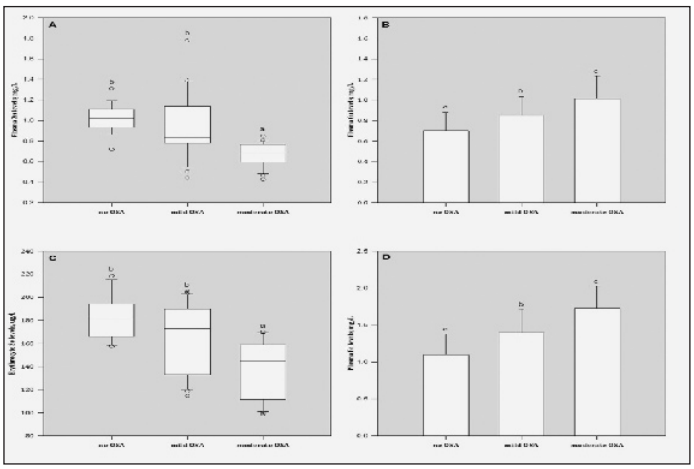


Figure 2

Correlations of AHI values with plasma concentrations of (a)hs-CRP, (b)TNF-α, (c)MDA, (d)erythrocyte GPx activity, (e)neck circumference in patients with mild to moderate OSA.
AHI = Apnea/hypopnea index; hs-CRP= high-sensitivity C-reactive protein; TNF-α= tumor necrosis factor-α;
MDA = malondialdehyde; GPx= glutathione peroxidase

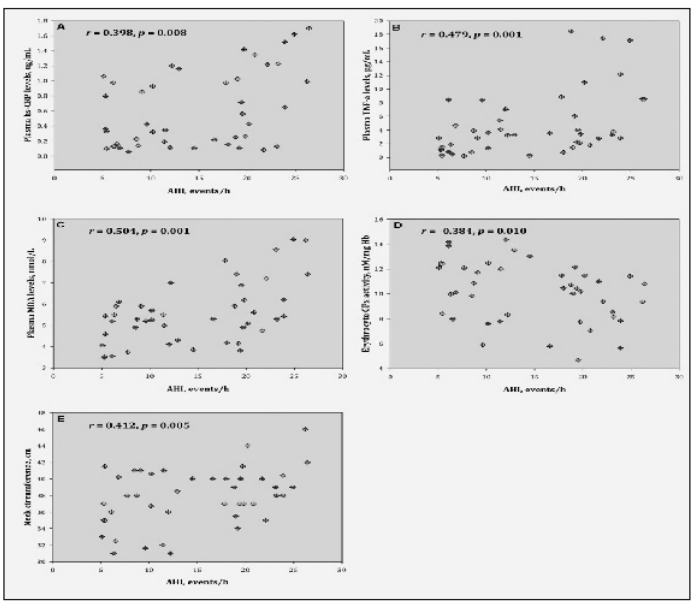
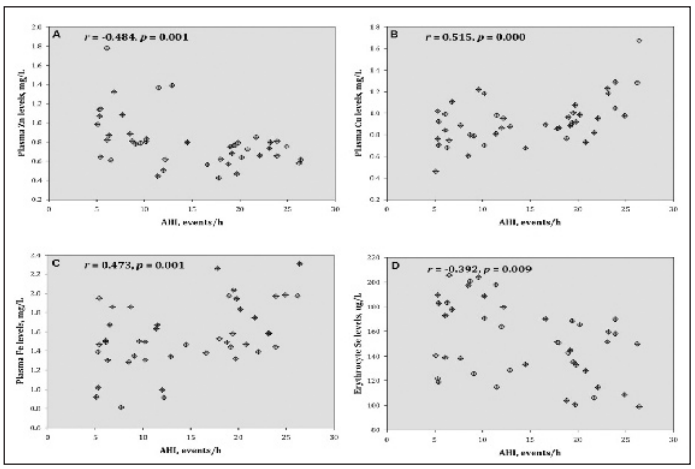


Figure 3

Associations between AHI values and blood variables (a)Zn, (b)Cu, (c)Fe, and (d)Se in patients with mild to moderate OSA.
AHI = Apnea/hypopnea index; Zn = zinc; Cu = copper; Fe = iron; Se = selenium



Discussion

In the present study, patients with moderate OSA had significantly abnormal blood levels of trace minerals (Cu, Zn, Fe, Se), higher MDA values (indicator of oxidative stress), lower activities of antioxidant enzymes (SOD and GPx), and increased hs-CRP and TNF-α levels compared to controls. In addition, we found that OSA severity, as indicated by the AHI values, was associated with the degree of altered inflammation, oxidative stress status, and disturbances of these trace minerals.

Our results showed a non-significant difference in BMI or waist circumference among patients with mild, moderate, and no OSA. Further, neck circumference, but not BMI or waist circumference, is positively associated with AHI values in OSA patients. Neck circumference has been suggested to be more predictive of OSA than general obesity or waist circumference (20, 21). The accumulation of adipose tissue around the pharynx may contribute to an increase in neck circumference.

OSA is a known cause of sleep-associated hypoxemia; thus, the high hemoglobin concentrations observed in patients with severe OSA may have been due to a compensatory need to carry more oxygen (17, 22). The present results showed no significant difference in hemoglobin concentrations between OSA patients and patients without OSA; whereas OSA patients had significantly higher oxygen desaturation.

In patients with untreated OSA, the episodes of hypoxia/reoxygenation during transient cessation of breathing are similar to hypoxia-reperfusion injury that cause the generation of ROS. These episodes are hence involved in the pathogenesis of OSA-related complications. Increased blood MDA, 8-isoprostane, neutrophil superoxide production, and urinary 8-hydroxy-2'-deoxy-guanosine have been reported in these patients (4, 7, 10, 23). Antioxidant enzymatic activities such as paraoxonase and arylesterase were also lower in OSA patients; whereas the degree of OSA severity was unknown

(24). The oxidative stress in OSA is related to BMI (25), while the severity of OSA is independently associated with oxidative stress (26). Further, an increased level of blood glucose can be associated with higher oxidative stress in OSA patients (27). Our results showed that, relative to controls of similar age, BMI, and blood glucose, mild to moderate OSA patients had significantly higher concentrations of MDA and lower antioxidant enzyme (SOD and GPx) activities. Taken together, these observations indicate that chronic intermittent hypoxia, as occurs in patients with even mild OSA, can promote oxidative stress.

Oxidative stress is involved in the activation of redox-sensitive transcription factors, which up-regulate target genes downstream of NF-ϰB. These target genes include pro-inflammatory cytokines such as interleukin-1, -6, and TNF-α (3, 25). Recent studies have found that the systemic inflammatory response, as measured by hs-CRP and TNF-α concentrations, was significantly higher in OSA patients compared to BMI-matched controls (28, 29). Pro-inflammatory cytokines can activate monocytes, lymphocytes, and endothelial cells, thus leading to endothelial dysfunction and cardiovascular disease. The hs-CRP can serve as a marker of upper airway inflammation in OSA patients (30), and plasma hs-CRP concentrations greater than 0.30 mg/dL bear an increased risk for cardiovascular morbidity (31). The present study shows that patients with mild to moderate OSA had a significantly higher oxidative stress and inflammatory status, which were associated with OSA severity.

An increasing number of studies suggest that OSA, alterations in oxidant-antioxidant balance and inflammatory responses are closely linked (32). The essential trace minerals Cu, Zn, Fe, and Se are key co-factors for regulating the expression of antioxidant enzymes and have immunomoderatory effects. Imbalances in trace minerals can lead to increased oxidative stress and a reduction in antioxidant enzymes. These changes induce oxidative damage, which is involved in the pathological processes that cause cardiovascular disease, diabetes, atherosclerosis, and chronic inflammation (33). We found that subjects with mild to moderate OSA had lower concentrations of plasma Zn and erythrocyte Se, and higher plasma concentrations of Cu and Fe compared to normal controls without OSA. Their alterations have also been associated with AHI values. This implies that abnormalities in trace minerals' homeostasis have deleterious effects on oxidative stress and inflammatory process and in turn may be implicated in the severity of OSA.

The cause for disturbances of trace mineral concentrations in these patients with mild to moderate OSA syndrome is unknown. In the present preliminary study, dietary intake data for micronutrients were not available, which we consider to be a limitation. A positive correlation has found between plasma CRP and matrix metalloproteinase (MMP)-9 concentrations, which is a Zn-containing endoprotease, in OSA patients (34). Up-regulation of Zn transporter genes in cells suffering oxidative damage can also reduce the plasma concentrations of Zn (35). Oxidative stress might be responsible for increased blood Cu concentrations (36). Cu may activate the phosphatidylinositol-3-kinase pathway, stimulate IL-6 production, and enhance the expression of intercellular adhesion molecule-1 (37). Excess Fe also accelerates ROS production and enhances TNF-α-induced endothelial cell activation, resulting in an increase in monocyte adhesion (38). A positive correlation between blood ferritin and hs-CRP concentrations has also been reported (39). Blood ferritin status was not available in our present work, although blood ferritin concentrations generally reflect Fe stores. Further, excessive Fe and Cu can interfere with Zn homeostasis, thereby contributing further to Zn deficiency (40). Evidence suggests that maintenance of adequate Se status plays a vital role in the prevention of hyperlipidemia and atherosclerosis (41, 42). Poor Se status is associated with increased oxidative stress and plasma IL-6 and TNF-α concentrations (43). Se also stimulates GPx enzyme activity and reduces transcription factor NF-ϰB activation (44). Altered concentrations of these trace minerals may aggravate the severity of OSA, oxidative stress, and inflammatory status; whereas mineral perturbations may be the consequence of inflammatory responses. Our clinical observation does not rule out a causal relationship in patients with mild to moderate OSA syndrome.

In conclusion, the present preliminary investigation found that patients with mild to moderate OSA syndrome had significant disturbances in homeostasis of the trace minerals Zn, Cu, Se, and Fe, which may contribute to an increased propagation of ROS and amplification of inflammatory processes. Therefore, disrupted homeostasis of these trace minerals is a potential risk factor for more severe OSA and its complications; improved trace mineral status may be useful to alleviate symptoms of OSA. Additional studies will be needed to clarify the exact roles of these trace minerals in the pathogenesis of OSA.

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Competing interests: The authors do not have any conflicts of interest to declare.

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