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ORIGINAL ARTICLES

Subfatin levels and thiol balance in patients with stroke

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Abstract

Background & Objective: Acute ischemic stroke leads to the increased production of free radicals and reactive oxygen species in the tissue and plasma via various mechanisms. We aimed to investigate thiol balance and subfatin impairment in patients with minor ischemic stroke (MIS) compared with stroke-free controls. *Methods:* A total of 70 participants (35 patients and 35 healthy controls) were enrolled in this study. The investigation includes serum subfatin levels, native thiol, total thiol, native thiol-to-total thiol ratio, disulfide, the disulfide-to-native thiol ratio, and the disulfide-to-total thiol ratio. The blood samples were collected at the time of admission to the emergency department. *Results:* A total of 35 patients with MIS and 35 healthy controls were enrolled in this study. Mean ages of patients and control subjects are 64.5±10.6 and 64.3±5.9, respectively. 20 (57.1%) of both groups were male. The two groups were similar in terms of age ($p > 0.912$) and did not differ in subfatin levels ($p = 0.247$). Native thiol, total thiol, and the native thiol-to-total thiol ratio were significantly lower in the patients than in the controls ($p < 0.001$), whereas disulfide, the disulfide-to-native thiol ratio, and the disulfide-to-total thiol ratio were significantly higher in the controls.
Conclusion: Thiol balance is impaired in patients with ischemic stroke, but it was uncertain about subfatin. Additional research required for subfatin in acute ischemic stroke.

Keywords: Acute ischemic stroke, disulfide, subfatin, thiol

INTRODUCTION

Acute strokes, also known as cerebrovascular accidents, are classified as either ischemic or hemorrhagic. Of the two types, acute ischemic stroke (AIS) results in the loss of blood flow, nutrients, and oxygen to particular areas of the brain, which causes neuronal damage and subsequent neurological deficits.[1] Acute ischemia leads to the increased production of free radicals and reactive oxygen species in the tissue and plasma via various mechanisms.[2] The results of several studies implicate a connection between oxidative stress and brain damage after ischemia and subsequent reperfusion related to AIS.[3] Although the exact mechanism of stroke remains unclear[4], oxidative stress is a pivotal event in the development of ischemic stroke and may contribute to stroke outcomes.[5]

Recently, studies have demonstrated that many well-known vascular risk factors such as hypertension, smoking, diabetes, cholesterolemia, atrial fibrillation, heart disease, and being overweight or obese are significantly associated with AIS in both the general population and among individuals at high risk of stroke.[6] In addition, newly identified risk factors include dyslipidemia, increased blood levels of C-reactive protein, carotid intima-media thickness, metabolic syndrome, old age, and gender have been identified. Even so, abdominal obesity, dyslipidemia, hyperglycemia, and symptomatic hypertension, collectively referred to as metabolic syndrome, are regarded as the four most important risk factors for ischemic stroke.[7]

Adipose tissue releases adipokines that play key roles in metabolic and cardio-cerebrovascular homeostasis.[8] Subfatin, also known as meteorin-like (i.e., metrnl) and cometin, is a blood circulating adipokine induced in muscles after exercise and adipose tissue upon exposure to cold that was recently identified as benefiting the metabolism of glucose. Increased circulating

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levels of subfatin stimulate energy expenditure, improve glucose tolerance, and influence the expression of genes associated with thermogenesis and anti-inflammatory cytokines.[9] It can also improve glucose tolerance and plays a crucial role in metabolism and both cardiovascular and cerebrovascular homeostasis.[8]

Thiols, are a class of organic compounds containing a sulfhydryl group (–SH) composed of a hydrogen and sulfur atom attached to a carbon atom that, in thiols, protects against oxidative stress. The plasma thiol pool is largely formed by albumin, protein thiols, and, to a lesser extent, low-molecular-weight thiols such as cysteinylglycine, cysteine, homocysteine, glutathione, and glutamylcysteine[10]. Thiols can undergo oxidation reaction via oxidants and form disulphide bonds.[11] When oxidative stress occurs, the oxidation of cysteine residues can lead to the reversible formation of mixed disulphides between protein thiol groups and low-molecular-mass thiols. The formed disulphide bonds can again be reduced to thiol groups; thus, dynamic thiol–disulphide homeostasis is maintained.[12] Tsai *et al*. found that, in the acute phase, patients with AIS had significantly lower free thiol levels than controls, that the free thiol was significantly lower in patients with large-vessel disease than in ones with small-vessel disease on Day 7 post-stroke, and that lower free thiol levels in the acute phase of AIS were associated with poor outcomes.[13]

In light of evidence indicating the pathophysiological role of oxidative stress in AIS and subfatin's role in metabolism and cerebrovascular homeostasis, we hypothesized that thiol balance and subfatin levels are impaired in patients with minor ischemic stroke (MIS) compared with individuals without the condition. To the best of our knowledge, our study was the first investigation of the correlation between subfatin and thiol–disulfide homeostasis in patients with MIS.

METHODS

Study design and participants

Our study was approved by the local ethics committee, and either patients or their next of kin signed their informed consent to participate. After the data were recorded per the Declaration of Helsinki, a patient number was assigned to each patient, and the patients' personal information was deleted. Based on radiographic imaging, ischemic infarction can be divided into either lacunar stroke or non-lacunar stroke, the former of which usually indicates MIS. However, the trial of ORG 10172 for acute stroke treatment revealed five subtypes of ischemic stroke, all of which may also contribute to MIS[14]; they include arterothrombotic MIS, cardioembolic MIS, and non-small vessel disease MIS due to another etiology.[15] Therefore, to be included in our study, patients with MIS had to be making their first visit to the emergency department of the hospital where we conducted the study with complaints of minor brain symptoms, with or without minor positive signs of stroke and measured as no more than 3 on the National Institutes of Health Stroke Scale. They also had to have magnetic resonance imaging displaying visible MIS within 24 h of initial presentation or else lacunar lesions or increased brain signal using diffusion-weighted imaging or fluid-attenuated inversion recovery, with a location in the subcortical white matter, the basal ganglia, or the brain stem. Patients with a history of stroke, progressive brain disease, chronic systemic disease, cancer, smoking, or alcohol consumption were excluded. Diagnosis was based on clinical data, neurological examination, and results of brain magnetic resonance imaging with diffusion-weighted imaging, a color duplex study of brain arteries, and transthoracic echocardiography. Meanwhile, controls were healthy individuals selected randomly from all individuals admitted to our hospital for checkup who did not have any known systemic diseases and were not taking any medications during the study same period.

Laboratory measurements

The participants' venous blood samples were collected at the time of admission to the emergency department and transferred into collection tubes. The samples were centrifuged at 5,000 × g for 10 min within 2 h, after which serum samples were separated and stored at –80 °C until analysis.

Subfatin assay

Subfatin levels were analyzed using YL Biont (Shangai, China) ELISA kits, which contain 96-well microplates pre-coated with human metrnl antibody. When subfatin in a sample was added to the 96-well microplate, it bound to the antibodies. After excess molecules were removed by washing, the biotinylated human metrnl antibody was added to bind to the captured subfatin of the sample. After HRP-streptavidin was added, it bound to the biotinylated subfatin antibody, and, following incubation, unbound streptavidin-HRP

was washed and removed. Subsequently, the substrate solution (i.e., tetramethylbenzidine) was added, and coloring emerged in proportion to the amount of subfatin. The reaction was terminated by adding a stop solution (i.e., sulfuric acid, 0.5 M), and absorbance was measured using a microplate reader (Cytation-1, BioTek, City, ST, USA) at 450 nm.

Dynamic thiol–disulfide balance assay

To determine the parameters of thiol–disulfide homeostasis, native thiol and total thiol levels were assayed using the spectrophotometric method described by Erel and Neselioglu.[16] Briefly, disulfide bonds were first reduced to form free functional thiol groups using sodium borohydride. The excess sodium borohydride reductant was removed with formaldehyde to prevent the reduction of DTNB, after which the thiol groups, including reduced and native thiol groups, were spectrophotometrically determined using the Cytation-1 microplate reader at 415 nm. Disulfide concentration and disulfide-to-native thiol ratios were calculated using the following formulas:

Disulfide levels (μmol/L) = (total thiol – native thiol) / 2

Disulfide-to-native thiol ratio (%) = (disulfide × 100) / native thiol

Because most serum thiols are formed by human serum albumin thiols (~80%), we calculated the adjusted total thiol, native thiol, and disulfide levels based on serum albumin concentrations using the following formulas:

Adjusted total thiol levels = total thiol (μmol/L) / albumin (g/L)

Adjusted native thiol levels = native thiol (μmol/L) / albumin (g/L)

Adjusted disulfide levels = disulfide (μmol/L) / albumin (g/L)

Statistical analysis

Data analysis was performed using SPSS version 25.0 (IBM Corporation, Armonk, NY, USA). The Shapiro–Wilk and Levene's tests were used to investigate whether the normal distribution and homogeneity of variance assumptions were met. Categorical data were recorded as numbers (n) and percentages (%), whereas quantitative data were recorded as M ± SD and median (i.e., 25th–75th) percentiles. Although mean differences between the patient group and control group were compared using Student's t test, the Mann–Whitney U test was applied to compare all continuous variables for which the parametrical test assumptions were not met. The optimal cutoff points of laboratory measurements to determine the existence of acute stroke were investigated by receiver operating characteristic (ROC) analyses and indicated the maximum sum of sensitivity and specificity for the significance test. Sensitivity, specificity, positive and negative predicted values, and accuracy rates for each significant laboratory measurement were also calculated. A p value less than .05 was considered to indicate statistical significance.

RESULTS

Seventy participants—35 patients and 35 healthy controls—were included in the study. The two groups were similar in terms of age ($p > .912$) and did not differ in subfatin levels ($p = 0.247$). Native thiol, total thiol, and the native thiol-to-total thiol ratio were significantly lower in the patients than in the controls ($p < 0.001$), whereas disulfide, the disulfide-to-native thiol ratio, and the disulfide-to-total thiol ratio were significantly higher in the controls. Age, subfatin, native thiol, total thiol, disulfide, the disulfide-to-native thiol ratio, the disulfide-to-total thiol ratio, and the native thiol-to-total thiol parameters of the groups and comparative results are presented in Table 1.

By contrast, Table 2 shows the results of the ROC analysis of the laboratory measurements in differentiating the control and patient groups.

Differentiating the groups, the areas under the ROC curves (AUC) of the measurements of subfatin measurements between the groups were not significant (AUC = 0.580, 95% CI: 0.441–0.720, $p = 0.247$); however, native thiol (AUC = 0.869, 95% confidence interval [CI]: 0.780–0.957), total thiol (AUC = 0.829, 95% CI: 0.725–0.932), and the native thiol-to-total thiol ratio (AUC = 0.946, 95% CI: 0.887–1.000) were statistically significant ($p < 0.001$) as shown in Figure 1. Further differentiating the groups, the AUCs of the measurements for disulfide (AUC = 0.847, 95% CI: 0.752–0.943), the disulfide-to-native thiol ratio (AUC = 0.938, 95% CI: 0.875–1.000), and the disulfide-to-total thiol ratio (AUC = 0.937, 95% CI: 0.874–1.000) were statistically significant ($p < 0.001$), as detailed in Figure 2.

Table 1: The comparisons of demographic characteristics and laboratory measures

	Controls (n=35)	Cases (n=35)	p-value
Age (years) *	64.3±5.9	64.5±10.6	0.912†
Male factor	20 (57.1%)	20 (57.1%)	N/A
Subfatin **	0.38 (0.35-0.53)	0.38 (0.36-0.42)	0.247‡
Native thiol *	328.7±65.8	244.4±51.7	**<0.001**†
Total thiol **	361.4 (342.3-398.6)	293.9 (276.3-331.8)	**<0.001**‡
Disulfide	17.2±5.9	24.7±5.7	**<0.001**†
Disulfide / Native thiol	5.5±2.4	10.3±2.2	**<0.001**†
Disulfide / Total thiol	4.9±1.9	8.5±1.5	**<0.001**†
Native thiol / Total thiol *	90.7±3.6	83.0±3.1	**<0.001**†

* Data were expressed as mean ± SD, ** Descriptive statistics were shown as median (25^{th} – 75^{th}) percentiles, † Student's t test, ‡ Mann Whitney U test. N/A: Not applicable.

Table 2: The ROC analysis results of laboratory indicators in distinguishing controls and cases from each other

	AUC	95% CI	p-value
Subfatin	0.580	0.441-0.720	0.247
Native thiol	0.869	0.780-0.957	**<0.001**
Total thiol	0.829	0.725-0.932	**<0.001**
Disulfide	0.847	0.752-0.943	**<0.001**
Disulfide / Native thiol	0.938	0.875-1.000	**<0.001**
Disulfide / Total thiol	0.937	0.874-1.000	**<0.001**
Native thiol / Total thiol	0.946	0.887-1.000	**<0.001**

AUC: Area under the ROC curve, CI: Confidence interval.

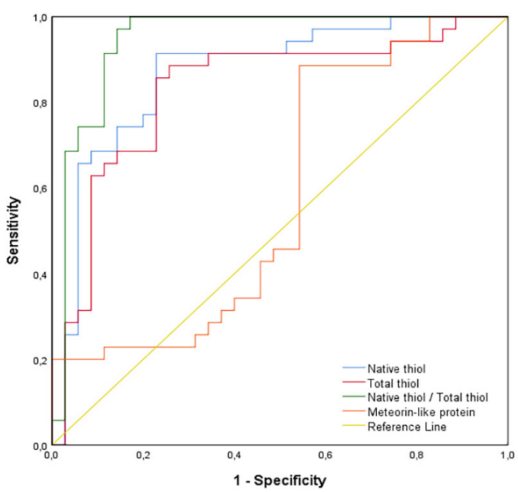

Figure 1. The AUCs of the measurements for native thiol, total thiol, native thiol-to-total thiol ratio and subfatin

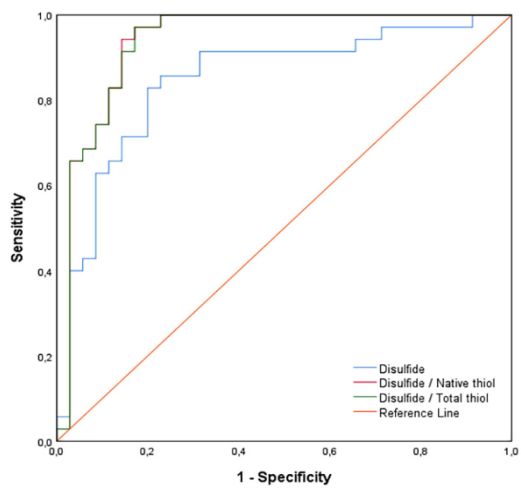

Figure 2. The AUCs of the measurements for disulfide, disulfide-to-native thiol ratio, and disulfide-to-total thiol ratio

Table 3 presents the best cutoff points for laboratory measurements where the AUCs are important and the diagnostic performance at those points distinguishing the control and patient groups.

DISCUSSION

In our study, we investigated the role of oxidative stress measured by thiol balance in patients with AIS, as well as the role of subfatin, a neurotrophic factor, in the incidence of acute cerebrovascular ischemic events. Our results showed a significant decrease in native thiol levels and total thiol among patients with AIS. The disulfide level was higher in the AIS group than in the control group. On the other hand, no significant difference was observed in subfatin levels between the two groups.

The subfatin protein, first described as a new neurotrophic factor in 2012[17], is primarily expressed by adipose tissue and skin. Subfatin acts via the Jak–STAT3 and MEK–ERK pathways to provide neuroprotection mediated by neurite outgrowth and neuroblast migration.[17,18] Because subfatin is a vital protein for maintaining cerebrovascular homeostasis function, we hypothesized that its levels during AIS might be affected. Thus, to our knowledge, our study was the first to involve evaluating subfatin levels in patients with AIS. In our analysis, we did not observe any significant differences between patients with AIS and controls in terms of subfatin levels. Possible explanations for that result include the low level of subfatin mRNA expression in the otic vesicle of medaka embryos.[18,19] Alternatively, restricted subfatin expression in the brain has been found during early mouse development when assessed by in situ hybridization, and a far lower level of expression in the adult mouse brain than in adipose tissue and skin has been observed.[19-21] In any case, further studies are therefore required to elucidate cerebrovascular function.

The dynamic thiol–disulfide equilibrium is essential for the antioxidative protection of cells[16], and the –SH group in thiols is especially thought to provide protection from oxidative damage. Some studies revealing the pathophysiological role of oxidative stress in AIS have indicated impaired thiol–disulfide homeostasis. In a study that involved assessing thiol, disulfide, and total thiol levels within the first 24 h after AIS, a substantial decrease was observed in favor of native and total thiol levels in patients with stroke compared with controls. However, in terms of disulfide levels, the two groups showed no significant differences.[10] The authors speculated that their finding indicates a maintained balance of thiol and disulfide concentrations. In a study, Yilmaz et al. evaluated thiol–disulfide homeostasis in patients with ischemic stroke and found that patients with AIS had considerably decreased native and total thiol values and higher concentrations of disulfide than the healthy controls.[22] Along similar lines, Icme *et al*.[23] compared the thiol levels of 92 stroke patients (i.e. 74 with ischemic and 18 with hemorrhagic stroke) with 75 volunteers and found that, in the ischemic group, thiol levels were significantly lower than among the controls. A study in Taiwan also confirmed that thiol levels were lower in patients with acute stroke 1 and 7 d after their ischemic events than for age- and sex-matched controls. In Italy, another study revealed that, within 24 h of AIS, –SH groups had decreased significantly.[24] Our findings are consistent with that literature, for patients with AIS in our study exhibited lower thiol levels and higher disulfide levels than the controls. In all studies, decreased –SH levels suggest an effort to counteract the aberrant production of free radicals.

Table 3: The optimal cut-off points for statistically significant laboratory measurements according to the ROC analysis and, diagnostic performances in distinguishing controls and cases from each other

	Cut-off point	Sensitivity	Specificity	PPV	NPV	Accuracy
Native thiol	<303.616	91.4%	77.1%	80.0%	90.0%	84.3%
Total thiol	<343.605	88.6%	74.3%	77.5%	86.7%	81.4%
Disulfide	>20.16	82.9%	80.0%	80.6%	82.4%	81.4%
Disulfide / Native thiol	>6.936	97.1%	82.9%	85.0%	96.7%	90.0%
Disulfide / Total thiol	>6.109	97.1%	82.9%	85.0%	96.7%	90.0%
Native thiol / Total thiol	<87.817	97.1%	85.7%	87.2%	96.8%	91.5%

PPV: Positive predictive value, NPV: Negative predictive value.

The importance of determining the thiol and disulfide levels in patients with AIS lies in the native thiol compound. Native thiol has hydrogen sulfide (H2S) as its functional group, which also happens to be the parent compound of nitric oxide (NO). Therefore, vascular toneus control is the primary physiological role of H2S, which promotes vasodilation by relaxing the endothelial cells, and vice versa, whereas decreased H2S levels diminish the vasodilatory effect. Because thiol levels fall during AIS, any treatment comprising a sulfhydryl group can raise the concentration of H2S, which may reduce neuronal damage caused by stroke and, in turn, enhance recovery.

Our findings have several limitations. First, our sample was small, and no power analysis was performed to determine the minimum sample size for the study. Second, we did not follow up with patients in order to monitor their long-term thiol–disulfide homeostasis. Third, no comparison was made between AIS and other oxidative stress markers, and antioxidant treatment effects were not assessed. Fourth, we included only mild cases of AIS, meaning that the role of thiol balance and subfatin in stroke severity remains to be clarified. Fifth and finally, confounders of increased oxidative stress were not included in our analysis.

In conclusion, although thiol–disulfide homeostasis may be used as a potential oxidative stress marker in patients with AIS, it is too premature to claim the same for subfatin. Considering the limitations of our findings, additional research is required to determine the prognostic significance of thiol and subfatin levels in AIS.

DISCLOSURE

Ethics: The study was approved by the ethics committee of Haran University (Decision number HRU/22.09.16).

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Conflict of interest: None

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