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Evaluations of Oxidative Stress, Thiol/Disulphide Homeostasis, and Nitric Oxide in Patients with Aneurysmal Subarachnoid Hemorrhage

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ABSTRACT

AIM: To investigate changes in nitric oxide (NO) levels, oxidative stress, and dynamic thiol/disulphide homeostasis in the serum and cerebrospinal fluid (CSF) of patients with aneurysmal subarachnoid hemorrhages (aSAH).

MATERIAL and METHODS: This prospective study included a total of 40 consecutive patients suffering from aSAH, who were operated on within the first 48 hours from onset of symptoms; CSF and blood samples were collected from these patients during their operations. To create a control group, blood samples and cerebrospinal fluid were taken from patients (n=40) without neurologic disorders who had undergone lumbar puncture for spinal anesthesia.

RESULTS: We observed that the serum total antioxidant status had decreased markedly ($p=0.0143$) but that no change was evident in the oxidative stress index and total oxidant status in aSAH patients when compared to the controls. While total thiol ($p=0.0014$) and native thiol ($p<0.0001$) levels had decreased in the aSAH patients, disulphide levels ($p<0.0001$) had increased significantly. Although the native thiol/total thiol ratio declined ($p<0.0001$), the dynamic disulphide/total thiol ratio ($p<0.0001$) and dynamic disulphide/native thiol ratio ($p<0.0001$) increased markedly in serum samples from the patient group. Patient serum NO levels were also significantly elevated ($p<0.0001$). There were no marked changes in CSF for all measured parameters ($p>0.05$).

CONCLUSION: This study demonstrated that serum NO levels and oxidative stress parameters increased markedly in the patients. These results may help to understand the underlying mechanisms behind early tissue damage due to aSAH and to monitor disease progression and improve the early detection of disease severity.

KEYWORDS: Aneurysm, Subarachnoid hemorrhage, Nitric oxide, Oxidative stress, Cerebrospinal fluid, Prediction, Progession

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a severe cerebrovascular disease associated with high morbidity and mortality (19). A ruptured aneurysm or traumatic head injury causes the extravasation of blood into the subarachnoid space (2,32). Survivors of SAH frequently have prolonged and disabling emotional and cognitive impairments, neurological deficits, and a reduced quality of life (19,32). Aneurysmal SAH (aSAH) has an estimated worldwide incidence rate between 5 and 10 per 100,000 person-years, with high regional variability (12).

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Accumulating evidence has suggested that oxidative stress is considered to be an important mechanism in the development of the pathophysiological process associated with aSAH (50,52); SAH refers to when a hemorrhage in the subarachnoid space increases intracranial pressure, thus inducing early brain injury and causing hypoxia and neural damage (18,29). In the acute phase of aSAH, oxyhemoglobin, released from blood components, causes the overexpression of peroxidase and mitochondrial dysfunction. This leads to excessive reactive oxygen species (ROS) formation that surpasses the body's antioxidant capacity, thereby generating early brain injury, including neuroinflammation and the disruption of the blood–brain barrier, inducing neuronal apoptosis, and causing long-term neurological dysfunction (50). Early brain injury is believed to occur in the first 72 h following ictus and is associated with the increased risk of delayed cerebral ischemia (2). Neuroinflammation appears to play a central role in the initiation of SAH, directly leading to endothelial and smooth muscle dysfunction as well as vessel wall damage, and is regulated by cytokines, chemokines, and ROS (43,50). Hemoglobin and oxyhemoglobin bind vasodilative substances, induce the formation of mediators causing vasoconstriction, and stimulate the generation of inflammatory agents such as cytokines and free radicals, thereby causing damage to the vascular endothelium and structural changes in smooth muscle cells (2,30).

Thiol/disulphide homeostasis is another commonly used modality to investigate the oxidant/antioxidant balance. ROS is able to convert thiols into disulphides via oxidation. The thiol/disulphide balance is stable in healthy individuals, but this dynamic balance is disrupted in favor of disulphide in the presence of SAH (1). Nitric oxide (NO) has been reported to be associated with the pathogenesis of cerebral injuries (6). The destruction of the NO pathway has been hypothesized to be a crucial mechanism underlying cerebral vasospasm and early brain injury (31). Thus, the oxidant/antioxidant balance and NO levels may contribute to the pathology of aSAH. Since oxidative stress plays an important role in pathological changes following aSAH, the purpose of this study was to determine whether there are differences in the serum and cerebrospinal fluid (CSF) concentrations of thiol/disulphide, total oxidant status (TOS), total antioxidant status (TAS), and NO levels between aSAH patients and controls.

MATERIAL and METHODS

Study Populations

This research involved a single-center prospective study of 40 consecutive patients with diagnoses of spontaneous single aSAH who had been admitted from January 2022 to August 2022 to the Department of Neurosurgery at Gaziantep University. This study received ethical approval from the Institutional Clinical Ethics Committee (Decision No and Date: 2022/16 and 26.01.2022); it was performed according to the Declaration of Helsinki, and written informed consent was obtained from the patients with full consciousness or from the first-degree relatives of patients without full consciousness prior to their participation. Two patients discontinued surgery due to severe bleeding, leaving 38 patients (19 men and 19 women) with an average age of 50.7±10.9 years in the aSAH patient group. The clinical features of all patients with aSAH, including their detailed medical history, laboratory findings, and demographics, were obtained. At the time of admission, the Hunt and Hess grading scale was applied for a clinical evaluation of the severity of aSAH (21). Patients were also graded according to the Glasgow Coma Scale (GCS) upon their admission (47). aSAH was confirmed via cerebral computerized tomography (CT) and digital subtraction angiography (DSA). aSAH was classified based on the pattern and distribution of hemorrhages according to the Fisher scale (13).

The inclusion criteria were as follows: >18 years of age; admission within 24 h of aSAH onset; confirmation of a ruptured aneuryms through DSA and CT angiography in SAH patients; no foreseeable early mortality due to brain stem injury; and the absence of contraindications to lumbar puncture.

The study excluded patients with chronic inflammation, severe infections, heart failure, autoimmune diseases, viral hepatitis, severe renal and hepatic failure, or malignant tumors; it also excluded those with a history of stroke, diabetes, or other systemic diseases; SAH with no clear source of hemorrhage according to cerebral CT angiography; or SAH accompanied with trauma complications.

All patients received standard medical care during their hospitalization. All patients underwent the coiling or clipping of the ruptured aneurysm within 48 h after SAH. The selection of an appropriate treatment modality (coiling or clipping) for each patient was governed by the neurosurgeon based on the size and location of the aneurysm; the selection was in accordance with the latest guidelines (5,19). After surgery, the patient's airway was kept open and unobstructed; their blood pressure was controlled; and their cerebral edema was treated or prevented. Standard medical treatment in line with the aSAH guidelines was provided to all patients, including neuroprotection, anti-vasospasm, acid–base balance, stress ulcer prevention, and nutritional support. Neurological changes in patients were carefully monitored during the hospitalization period. The appearance of a focal neurologic deficit, the insidious onset of confusion, or both, were regarded as symptomatic of vasospasm. Cerebral CT scans were performed to rule out hemorrhages, acute ischemic events, or the occurrence of acute hydrocephalus.

A total of 40 patients served as controls, including 31 with lumbar disc diseases and nine with normal pressure hydrocephalus. The inclusion criteria for controls were as follows: >18 years of age; no inflammatory disease or systemic disease; no prior history of strokes; no drug/substance dependence or history of alcohol abuse; and the presence of a tumor.

Blood and CSF Samples

Blood samples and CSF were collected at the time of surgery. Blood specimens were collected in plain tubes and incubated to clot for 20 minutes at 4°C. All the blood and CSF samples were centrifuged (1500 *g*, 10 mins, at 4°C) immediately after collection and were then separated into suitable aliquots and frozen at -80°C for further assays. All biochemical measurements were performed by specialist blind to study groups.

Thiol/disulphide Measurements

Thiol/disulphide levels were analyzed according to the previously published methods (36). Commercial kits (Rel Assay Diagnostics, Mega Tip Ltd, Gaziantep, Turkey) were used to measure the total thiol (–SH + –S–S–) and serum native thiol (–SH) levels. In this method, the disulphide bond was reduced to free functional thiol groups using sodium borohydride ($NaBH_4$). Unused $NaBH_4$ was reacted and discarded with formaldehyde. Total thiol and native thiol levels were measured after reactions with dithionite-2 nitrobenzoic (DTNB), and their levels were determined spectrophotometrically. Half of the difference between the total thiols and native thiols formed the dynamic disulphide (–S–S–) value.

Total Antioxidant Status (TAS)

The serum TAS level was analyzed using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey). This method uses 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cations. Antioxidants in the samples are capable of bleaching the characteristic color of a stable ABTS cation. The data are presented as the mmol Trolox equivalent per liter (10).

Total Oxidant Status (TOS)

The serum TOS level was analyzed using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey). The oxidants present in the serum samples oxidize the Fe^{2+}-o-dianisidine complex to the Fe^{3+} ion. The Fe^{3+} ion generates a colored compound in reaction to xylenol orange in an acidic condition. The color intensity of xylenol orange is directly proportional to the oxidant levels and is measured spectrophotometrically. Absorbance demonstrates the total amount of oxidant molecules present in the specimen. For the calibration, hydrogen peroxide (H_2O_2) was utilized, and the data are indicated based on the μmol H_2O_2 equivalent per liter (11).

Oxidative Stress Index (OSI)

The ratio of TOS to TAS is accepted as the OSI, as described previously (16). The OSI value was determined according to the following formula: OSI (arbitrary unit) = TOS (μmol H_2O_2 equivalent/L) / TAS (μmol Trolox equivalent/L).

NO Analysis

Concentrations of NO in samples were measured using chemiluminescence-based assay, as reported previously (25). Following deproteinization with absolute ethanol treatment (at 0°C in a 1:2 v/v mix), the samples were incubated for 30 min at 0°C and then centrifuged at 20800 *g* for 5 min. The NO analyzer (Model 280i NOA, Sievers Instruments, Boulder, CO, USA) was used to measure the NO levels from the supernatants. Vanadium trichloride (dissolved in 1 M HCl at 95°C) was utilized to reduce all NOx species (NO_2^- + NO_3^- + SNO^-) to NO, and the NO formed was measured in the presence of pure nitrogen gas. Sodium nitrite standards were used to quantify the NO levels. The NO analysis software was utilized for data collection and analysis.

Statistical Analysis

The data are presented as the mean ± the standard deviations (S.D.) and were analyzed using GraphPad Instat (version 3.05, GraphPad Software Inc., San Diego, CA, USA). The qualitative data are expressed as ratios with percentages. The normality of the distribution was analyzed using the Kolmogorov–Smirnov test. A Student's t-test and a Mann–Whitney U test were used for group comparisons of normally and abnormally distributed variables, respectively. A chi-squared test was utilized for the comparison of categorical data. Spearman's or Pearson's tests were used to evaluate the correlation analyses. A P-value of more than 0.05 is considered statistically significant.

RESULTS

Clinical and laboratory parameters of the study groups are summarized in Table I. There were no meaningful differences in age, gender, hemoglobin, body mass index, white blood cells, platelet count, sedimentation, creatinine levels, or smoking status between the patients and control groups ($p>0.05$ for all). However, there was a marked elevation in C-reactive protein levels ($p<0.0001$). Thirty-four patients with intracranial aSAH underwent microsurgical clipping, and four patients underwent coiling. Acute hydrocephalus was present in two patients. Glasgow Coma Scale (GCS) scores upon admission ranged from 8 to 15. Serum TAS values were markedly attenuated in the patient group ($p<0.05$, Figure 1). However, no changes were recorded for TOS and OSI. Although serum total thiol and native thiol levels were depressed, disulphide levels were markedly augmented in the patient group (Figure 2). There were significant reductions in the native thiol/total thiol ratio and disulphide/total thiol ratio, but we noted marked augmentation in the disulphide/native thiol ratio (Figure 3). Serum NO levels were also augmented in the patient group ($p<0.0001$, Figure 4). However, no marked changes in CSF levels were observed for all measured parameters.

Correlation analyses revealed that there were negative correlations between GCS and the Fisher scale or Hunt and Hess grading, as shown in Table II. However, there were positive correlations between Hunt and Hess grading and the Fisher scale or TOS. A marked positive correlation was noted between NO levels and native or total thiol. Additionally, total thiol exhibited positive correlations with disulphide and native thiol levels (Table II).

DISCUSSION

In this prospective study, we assessed the CSF and serum thiol/disulphide, TAS, TOS, and NO levels of aSAH patients and compared them with those of the controls. We documented that serum NO and disulphide levels were markedly augmented in the aSAH group when compared to the controls. We noted significant reductions in serum TAS, total thiol, and native thiol levels in the aSAH group. Although this is the first study to examine the CSF levels of thiol/disulphide in aSAH patients, no marked changes were observed. Taken together, these findings demonstrate that serum (but not CSF) levels of oxidative stress parameters could be a better indicator of the pathophysiological process observed in aSAH.

Table I: Demographic, Laboratory and Clinical Features of Patients with aSAH and in Controls

	Control (n=40)	Patients with aSAH (n=38)	p-value
Age (years)	48.7±18.7	50.7±10.9	0.5682
Gender			0.8296
Male (n %)	22 (55.0)	19 (50.0)	
Female (n %)	18 (45.0)	19 (50.0)	
Height (cm)	169.7±10.7	168.5±10.6	0.6204
Weight (kg)	76.3±18.4	79.2±11.0	0.4039
Body mass index (kg/m^2)	26.6±6.1	27.9±3.5	0.2552
Hemoglobin (g/dl)	14.1±3.9	13.9±2.1	0.7803
White blood cells (x 10^3/mm^3)	11.9±2.9	12.8±4.7	0.3093
Platelet count (x 10^3/mm^3)	271.3±68.2	260.5±84.5	0.5354
INR	-	1.1±0.1	-
Sedimentation (mm/h)	14.6±4.9	13.2±9.0	0.3930
C-reactive protein (mg/L)	0.6±0.3	6.5±5.1	**<0.0001**
Creatinine (mg/dl)	0.8±0.3	0.7±0.2	0.0890
Na^+ (mmol/L)	-	137.2±3.8	
K^+ (mmol/L)	-	3.9±0.5	
Smoking status (n, %)			1.0000
Yes	7 (17.5)	6 (15.8)	
No	33 (82.5)	32 (84.2)	
Comorbidity (n, %)			
Hypertension	-	13 (34.2)	
Heart valve disease	-	1 (2.6)	
Coronary artery disease	-	1 (2.6)	
Asthma	-	1 (2.6)	
Hypothyroidism	-	1 (2.6)	
GCS	-	11.9±2.5	
Fisher scale	-	2.9±0.9	
Hunt and Hess grading	-	2.6±1.0	

Data show mean ± SD values. ***INR:*** *International normalized ratio,* ***GCS:*** *Glasgow coma scale.*

Increased levels of superoxide radicals in the CSF after aSAH have been reported to have a connection with cerebrovascular spasm, which may further cause cerebral ischemia and elevated intracerebral pressure (14). Following SAH, blood-derived hemoglobin and oxyhemoglobin in the subarachnoid space bind NO and therefore inhibit its vasodilating effect. Oxyhemoglobin-induced vasoconstriction is involved in the depression of voltage-dependent K^+ channels, NO depletion, and the upregulation of R-type Ca^{2+} channel expressions in the cerebral vascular system (23,30,40). Moreover, oxidative stress-induced damage to the DNA, proteins, and lipids can result in cytotoxic edema and the subsequent increase of intracerebral pressure as well (14,17). High malondialdehyde levels in the CSF of patients with aSAH have been detected (4). We demonstrated that serum TAS levels were markedly reduced in aSAH patients. Marzatico et al. also demonstrated in rats that the antioxidant capacity of antioxidant enzyme systems is depressed after experimental SAH (34). This could be the reason for the observation of negative effects on antioxidant stress following SAH. The suppression of superoxide

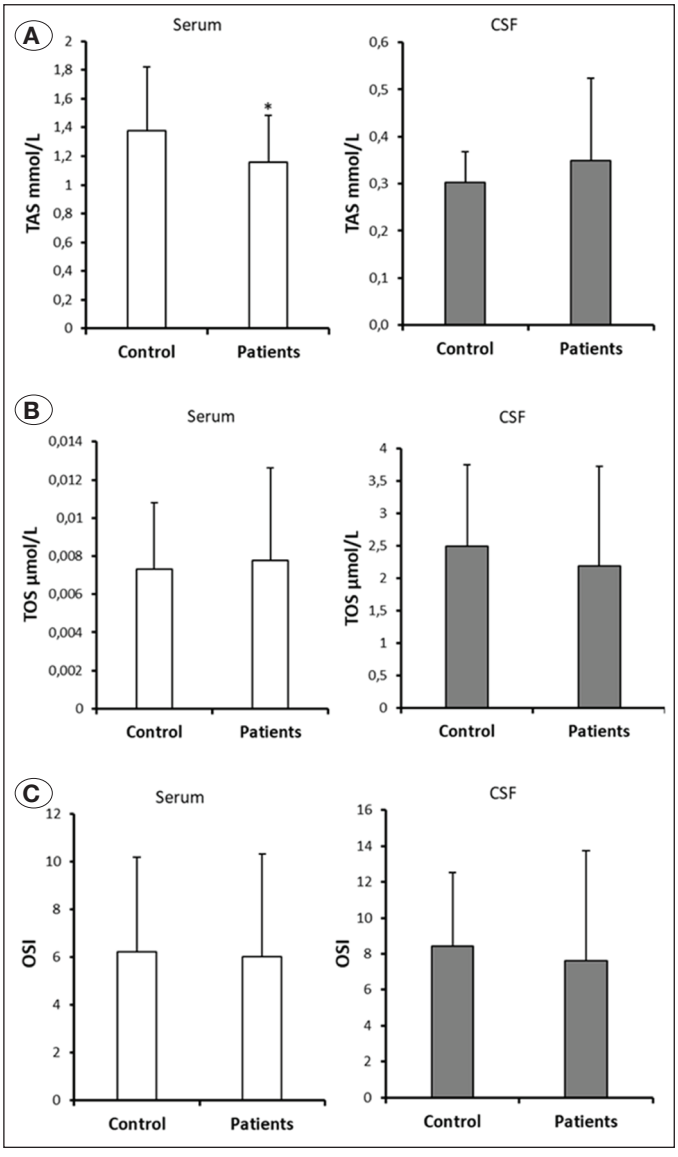

Figure 1: Serum and cerebrospinal fluid (CSF) total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) in the control (n=40) and patient (n=38) groups. Values are indicated as mean ± SD, *p=0.0143 when compared to controls.

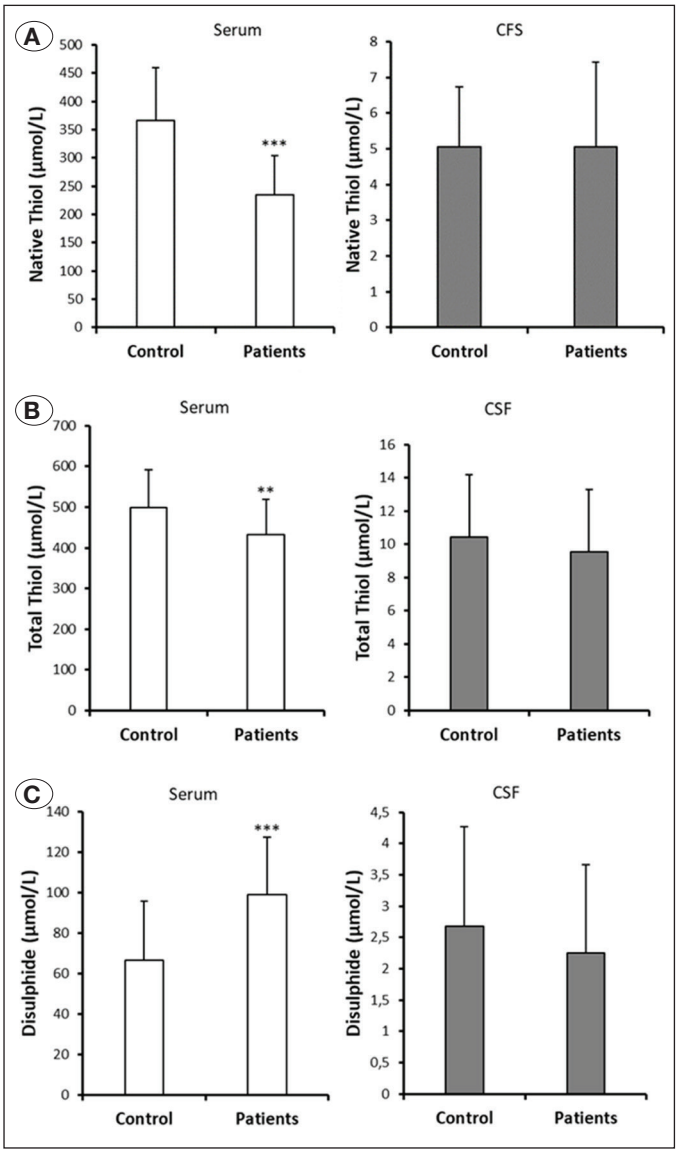

Figure 2: Serum and cerebrospinal fluid (CSF) native thiol, total thiol, and disulphide levels in the control (n=40) and patient (n=38) groups. Values are provided as mean ± SD, **p=0.0014, ***p<0.0001 when compared to controls.

dismutase (SOD) enzymes in CSF and plasma have been reported to be linked to long-term poor neurological outcomes after aSAH (28). Kaynar et al. demonstrated that the mean CSF SOD levels were lower and that serum malondialdehyde levels were higher than the controls, suggesting that the levels of antioxidants are diminished after the onset of SAH, possibly because of increased oxidative stress (26). Taken together, we postulate that the aSAH resulted in the excessive generation of oxidative stress, which may cause various neuropathologic changes during brain injury.

We demonstrated decreased serum total and native thiol levels and augmented disulphide levels in aSAH patients. These findings are consistent with the previous study published by Abdallah et al. (1). In fact, there is only one published study evaluating the role of thiol/disulphide homeostasis in patients with aSAH (1). We observed that there was a diminished TAS value but no changes in OSI and TOS values in aSAH patients. However, Abdallah et al. described augmented TOS and OSI and suppressed TAS values in patients with aSAH (1). Our data pertaining to decreased TAS values were supported by this study. We found no correlation between the OSI and the thiol/disulphide values, as demonstrated by Abdallah et al. (1). However, we observed positive correlations between Hunt and Hess grading and TOS. We also revealed that there were positive correlations between NO levels and native thiol or total thiol levels. Notably, antioxidant vitamin E was administered daily to all patients in the study performed by Abdallah et al., which might have influenced the results (1).

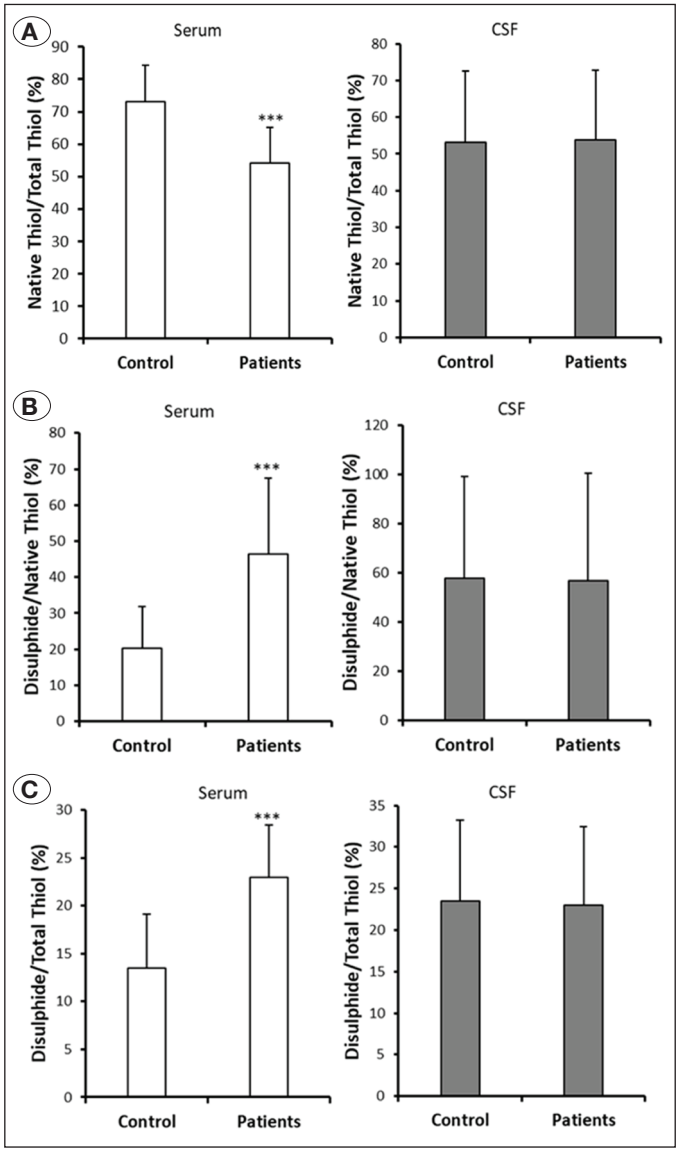

Figure 3: Serum and cerebrospinal fluid (CSF) native thiol/total thiol ratio, disulphide/native thiol ratio, and disulphide/total thiol ratio in the control (n=40) and patient (n=38) groups. Values are presented as mean ± SD, ***p<0.0001 when compared to controls.

We found that serum (but not CSF) NO levels were markedly augmented during surgery in aSAH patients. Our findings related to CSF NO levels are supported by the results indicating that the CSF nitrite levels determined in the control group were similar to levels in the SAH group on Days 0 (24). However, our results related to NO are not consistent with those of Ramesh et al., who showed that the plasma concentrations of the NOx were markedly reduced in the patients group (39). This difference in NO levels may be related to the collection time of the blood samples. We collected blood samples during the operations, but Ramesh et al. collected samples at the time of admission of each patient (39). After SAH, hemoglobin and oxyhemoglobin bind NO and cause a loss of NO. However, Yatsushige et al. revealed that NO exceeded baseline values within 24 h after SAH and remained elevated thereafter (51). However, the immunological response after SAH, particularly following the interaction of the vascular tissues with oxyhemoglobin, induces a reactive augmentation in the inducible form of NO synthase (iNOS) expression in activated microglia and macrophages. Upregulated iNOS induces the overproduction of NO, which appears to be responsible for NO-induced secondary injuries after SAH (22). A high level of NO may cause the peroxidative injury of cell membranes,

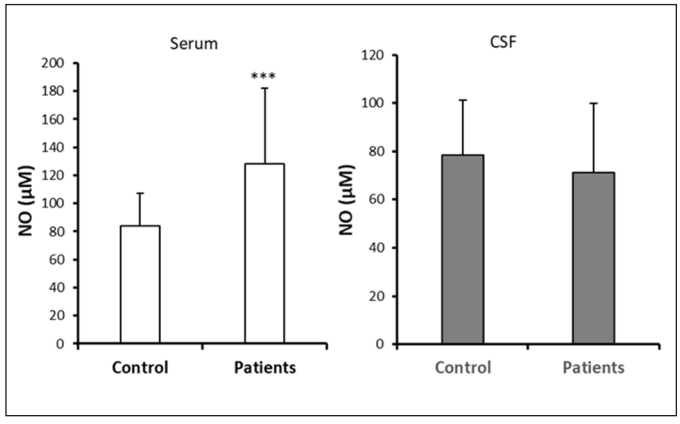

Figure 4: Serum and cerebrospinal fluid (CSF) nitric oxide (NO) levels in the control (n=40) and patient (n=38) groups. Values are indicated as mean ± SD, ***p<0.0001 when compared to controls.

Table II: Correlations Between Thiol/Disulphide Homeostasis, Scales/Grades, and Serum NO Levels in Patients with aSAH

Parameters	Correlation coefficient (r)	Coefficient of determination (r^2)	p-value
GCS ↔ Fisher scale	-0.7069	0.4997	**<0.0001**
GCS ↔ Hunt and Hess grading	-0.9008	0.8114	**<0.0001**
Fisher scale ↔ Hunt and Hess grading	0.6851	0.4693	**<0.0001**
Hunt and Hess grading ↔ TOS	0.3484	0.1214	**0.0320**
NO ↔ Native Thiol	0.4138	0.1712	**0.0167**
NO ↔ Total Thiol	0.4270	0.1823	**0.0132**
Native Thiol ↔ Total Thiol	0.7476	0.5589	**<0.0001**
Total Thiol ↔ Disulphide	0.5971	0.3566	**<0.0001**

GCS: *Glasgow Coma Scale;* ***TOS:*** *Total oxidant status;* ***NO:*** *Nitric oxide.*

causing a pathological modification of the arterial smooth muscle and the endothelial cell layers. Thus, the peroxidation of membrane proteins by the NO metabolite peroxynitrite could most likely contribute to the morphological damage observed in chronic vasospasms (35). The contribution of CSF NO levels to the development of the aSAH process is controversial. Sakowitz et al. documented that there were decreases in microdialysate NOx levels on day 7 after SAH (42). Marginally reduced levels of NO metabolites in the CSF of SAH patients compared to healthy controls have been identified (8). Martens-Lobenhoffer et al. reported a simultaneous reduction in nitrate and nitrite levels in CSF and suggested that there is a progressive impairment of the NO formation rate in the brain following SAH (33). A preliminary report found that intrathecally administered sodium nitroprusside, an NO donor, compensated for the decreased availability of NO and caused the reversal of cerebral vasospasms in aSAH patients (48). It has been reported that treatment with two NO donors, namely via intravenous molsidomine infusion and intraventricular boluses of sodium nitroprusside, could reverse vasospasm and exert a positive impact on outcomes in patients with severe aSAH (9). A prospective pilot study evaluating the effects of inhaled NO on cerebral perfusion in patients with delayed cerebral ischemia after aSAH revealed that the administration of inhaled NO in SAH patients is safe, but it suggested that conclusions regarding the general applicability of inhaled NO in SAH patients are not possible (15). In contrast to these observations, various studies in humans have analyzed NO levels after SAH within the CSF (37,38,45,46) and cerebral interstitial fluid (i.e., microdialysate) (37,41,44), and elevated levels of NO have been reported. NO levels in CSF have been found to peak within the first five days following SAH (37). Woszczyk et al. indicated that SAH causes an increase in NO metabolites in CSF during the follow-up period between day 2 and 8, implying that NO is crucial in the pathogenesis of cerebral vasospasm (49). In support of this data, Hosmann et al. demonstrated that cerebral microdialysate NO levels generated biphasic kinetics with drastically augmented levels during the first seven days and significantly lower levels within eight to 14 days in aSAH patients (20). NO overproduction, induced by various factors, could stimulate the production of peroxynitrite and free radicals, which induce damage to the cell structures (7). Such morphological modifications in the vascular structure could lead to endothelial dysfunction and vasoconstriction. Additionally, the increase in NO levels is capable of impairing mitochondrial function during the acute phase of aSAH. Indeed, excessively elevated NO plays a pivotal role in neuronal damage, generating mitochondrial dysfunction and facilitating the accumulation of mitochondrial substrates (3,20). Kho et al. suggested that elevated NO levels in CSF and serum and reductions in NO in the CSF-to-serum ratio were associated with the severity and occurrence of vasospasm and clinical outcomes in aSAH patients (27). Serum NO levels were statistically significantly higher in patients with high severity gradings and with radiological vasospasm (27). This indicates the possible role of NO as a biomarker to assess severity and prognosis in patients with SAH, which is consistent with our results. Taken together, these results imply that NO is heavily involved in the pathogenesis of the acute phase of aSAH.

The main limitation of our study is the small sample size. It appears that it is essential to obtain clinical and biochemical values from larger patient cohorts for an enhanced understanding of the role of oxidative/nitrosative stress.

CONCLUSION

Our results demonstrated that thiol/disulphide homeostasis play a pivotal role in pathological processes following aSAH. Moreover, plasma NO and thiol/disulphide levels can be used as candidate biomarkers for predicting outcomes in aSAH patients. Since the early determination and treatment of intracranial aneurysms can remarkably improve the survival rates of patients with aSAH, our findings may help enhance therapeutic strategies involving the reduction of oxidative stress in the early stages of aSAH.

Declarations

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Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure: Authors declare no conflict of interest.

AUTHORSHIP CONTRIBUTION

Study conception and design: BKU, AMG, ATD

Data collection: NT, AN, BKU, AMG, NU

Analysis and interpretation of results: AS, SD, ATD

Draft manuscript preparation: SD, ATD

Critical revision of the article: BKU, SD, ATD

Other (study supervision, fundings, materials, etc...): BKU, AMG, ATD

All authors (NT, AN, BKU, AMG, NU, AS, SD, ATD) reviewed the results and approved the final version of the manuscript.

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