

Original Research Article

The role of oxidants and antioxidative parameters in patients with hepatic encephalopathy

Sami Bahcebasi^{1*}, Gulden Baskol², Mevult Baskol³

¹Department of Internal Medicine, Kayseri City Hospital, Turkey

²Department of Biochemistry, Erciyes University, Turkey

³Department of Gastroenterology, Erciyes University, Turkey

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*Correspondence:

Sami Bahcebasi,

E-mail: doktorsami@yahoo.com

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ABSTRACT

Background: Hepatic encephalopathy is a serious neuropsychiatric complication of cirrhosis. Changes in the oxidative and anti-oxidative system and nitric oxide levels in brain tissue contribute to the development of symptoms related to HE and HE. Purpose of the study to reveal the alterations in oxidative, anti-oxidative system and nitric oxide levels in cirrhotic patients during and after hepatic encephalopathy periods.

Methods: This was a randomized controlled double-blind study conducted in Erciyes University Hospital between 3 July 2010 and 30 March 2011. We investigated the oxidative and anti-oxidative stress parameters by quantification of total antioxidant capacity (TAC), total oxidant capacity (TOC), nitric oxide (NO), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total thiol and xanthine oxidase (XO) levels in serum. We compared the group of patients with hepatic encephalopathy, post-hepatic encephalopathy (clinically recovered) and control groups (healthy control).

Results: Thirty hepatic encephalopathy patients were studied. Serum levels of nitric oxide and xanthine oxidase were statistically significantly high in the hepatic encephalopathy group according to control group ($p < 0.031$, and $p < 0.001$, respectively). Serum thiol levels were significantly low in hepatic encephalopathy patients than the controls ($p < 0.001$). Total oxidant capacity, total antioxidant capacity, glutathione peroxidase and superoxide dismutase levels were not significantly different in hepatic encephalopathy group than the controls. Serum thiol levels were low and serum NO levels were high in recovered clinically from hepatic encephalopathy group according to control group currently ($p < 0.001$, $p < 0.001$, respectively). Total antioxidant capacity, total oxidant capacity, glutathione peroxidase, superoxide dismutase and xanthine oxidase levels were similar in both groups ($p > 0.05$). Total antioxidant capacity and especially xanthine oxidase levels were significantly decreased in recovered clinically from hepatic encephalopathy group compared to hepatic encephalopathy group ($p < 0.05$, $p < 0.001$, respectively).

Conclusions: Oxidative system, in systemic circulation, is activated during hepatic encephalopathy and changes in XO level during and after hepatic encephalopathy is very different. This parameter may be a potential marker in differential diagnosis of hepatic encephalopathy from other coma causes. Further investigation is needed.

Keywords: Hepatic encephalopathy, Oxidative stress, Xanthine oxidase

INTRODUCTION

Hepatic encephalopathy (HE) is a complex neuropsychiatric syndrome present in patients with acute

or chronic liver disease.¹ There is consensus that ammonia is a key toxin in HE, which may sensitize the brain to the different precipitating factors.² The term oxidative stress refers to a condition in which cells are subjected to

excessive levels of molecular oxygen or its chemical derivatives, namely reactive oxygen species (ROS), and they are unable to counterbalance their deleterious effects with antioxidants.³ ROS are natural by-products of oxygen metabolism and include oxygen ions, free radicals, and peroxides, both inorganic and organic. ROS are small molecules and are highly reactive due to the presence of unpaired electrons. During times of oxidative stress, ROS accumulate to toxic levels, resulting in cellular damage and compromised function.⁴ In clinical studies, systemic oxidative stress has been shown in cirrhotic patients with HE, whereas it has been shown that there is no oxidative stress in cirrhotic patients without encephalopathy.⁵

There is substantial evidence from animal and cell culture studies that oxidative/nitrosative stress plays an important role in the pathogenesis of hepatic encephalopathy and ammonia toxicity. In a study in cultured astrocytes and rat brain, it was shown that ammonia, inflammatory cytokines, benzodiazepines, and hyponatraemia induce rapid formation of reactive oxygen and nitrogen species, including nitric oxide (NO).⁶⁻⁸ There is close relationship between astrocyte swelling and oxidative stress. Astrocyte swelling induces oxidative stress through NMDA receptor-dependent and Ca²⁺ dependent mechanisms. On the other hand, NMDA receptor activation and oxidative stress trigger astrocyte swelling. These points to an auto-amplificatory signaling loop between astrocyte swelling and oxidative stress.^{9,10} Although cell culture studies in vitro and rodent models in vivo suggest an important role of oxidative/nitrosative stress in the pathogenesis of ammonia toxicity, it was unknown whether oxidative stress is also involved in the pathogenesis of HE in humans with cirrhosis. Recently published study clearly demonstrated the direct evidence of oxidative/nitrosative stress in post mortem brain tissues of hepatic encephalopathy patients.¹¹ Human brain studies in encephalopathy patients are nearly impossible because of ethical and technical issues. In addition to that there is no comprehensive study investigating the alterations that occur in systemic oxidative and anti-oxidative system in serum samples of hepatic encephalopathy patients. Antioxidant and zinc supplementation improve minimal HE in patients with liver cirrhosis.¹²

Therefore, these findings indicate that oxidative stress plays a role in the development of astrocyte swelling, brain edema, and HE. There is increasing evidence of oxidative/nitrosative stress in the pathogenesis of HE due to acute or chronic liver failure in experimental brain models, and several reports indicate that oxidative/nitrosative stress plays a role in the pathophysiological cascade responsible for HE.¹³ Gorg et al analyzed post mortem cortical brain tissue samples from patients with cirrhosis dying with or without HE in comparison with brains from patients without liver disease and concluded that HE in patients with cirrhosis is associated with oxidative and nitrosative stress.¹⁴ Cell culture studies in vitro and rodent models in vivo suggest an important role of oxidative/nitrosative stress in the

pathogenesis of ammonia toxicity. Free radicals are produced in metabolic and physiological processes, and harmful oxidative reactions may occur in organisms. Organisms are protected against oxidative stress via enzymatic and nonenzymatic antioxidative mechanisms. Kosenko et al and Hilgier et al have shown that the infusion of ammonium into the corpus striatum of rats results in the production of hydroxyl radicals.^{15,16}

Kosenko et al reported a significant decrease in the activity of antioxidant enzymes, as well as increased lipid peroxidation and reduced glutathione in these rats brain.¹⁵ We investigate the oxidative parameters by quantification of total oxidant capacity (TOC), nitric oxide (NO), and xanthine oxidase (XO) levels. Anti-oxidative parameters; total antioxidant capacity (TAC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and total thiol, levels in serum of hepatic encephalopathy patients. In this study, we aimed to find the variations in the oxidative and antioxidative systems in patients with hepatic encephalopathy, in the recovery period and in healthy controls by taking serum samples, which is a non-invasive method. Different parameters have been investigated.

METHODS

This was a randomized controlled double-blind study conducted in Erciyes University Hospital between 3 July 2010 and 30 March 2011.

Subjects

Thirty cirrhotic patients (19 male and 11 female) with HE (80% child C and 20 % child B) and 31 healthy subjects (17 male and 14 female) were examined. West Hevan Criteria was used to grade HE status.¹⁷ Four (13, 3%), 13 (43, 3%), 9 (30%), and 4 (13, 3%) patients were classified as grade 1, 2, 3 and 4 respectively.

Four groups were formed as HE groups, patients who recovered clinically HE episode were named as post hepatic encephalopathy group (PHE), HE patients who did not survive during hospitalization period were named as ex group (EX) and healthy volunteers were named as control group. Patients treated according to identification-correction of precipitating causes and measures to lower blood ammonia concentration.

Erciyes University Institutional Review Board for Human studies approved this study (TSU-11-3422) and Erciyes University scientific project department gave financial support to the study.

Samples

Serum samples were withdrawn at intensive care department after written consent form was taken from first-degree relatives of patients. Second serum samples were withdrawn after recovery. Serum samples were stored at -80°C until being analyzed.

Chemicals

All chemicals used in this study were from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade or the highest grade available.

Biochemical analysis

Assay of total oxidant capacity

TOC levels were measured using commercially available kits (Rel assay). In the new method, oxidants present in the sample oxidized the ferrous ion-o-dianisidine complex to ferric ion.¹⁸ The oxidation reaction was enhanced by glycerol molecules abundantly present in the reaction medium. The ferric ion produced a colored complex with xylenol orange in acidic medium. The color intensity, which could be measured spectrophotometrically, was related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromolar hydrogen peroxide equivalent per liter ($\mu\text{mol H}_2\text{O}_2$ equivalent/l).

Assay of NO levels

NO levels were performed following the methods of Green LC et al using the Cayman's nitric oxide assay kit.¹⁹ The results are presented in $\mu\text{mol/l}$.

Assay of XO activity

Serum XO activity was measured according to the method of Prajda and Weber, where the activity is measured by determination of uric acid from xanthine. Plasma (50 ml) was incubated for 30 min at 37°C in 3 ml of phosphate buffer (pH 7.5, 50 mM) containing xanthine (4 mM).²⁰ The reaction was stopped by addition of 0.1 ml 100% (w/v) TCA, the mixture was then centrifuged at 4000 g for 20 min. Urate was determined in the supernatant by measuring absorbance at 292 nm against a blank and expressed as units per milliliter (U/ml) in serum.

Assay of total antioxidant capacity

TAC levels were measured using commercially available kits (Rel assay). The method is based on the bleaching of characteristic color of a more stable ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation by antioxidants.²¹ The results were expressed as mmol Trolox (Rel assay) equivalent/l.

Assay of GSH-Px activity

GSH-Px activity in serum was measured according to the method of Paglia and Valentine.²² Enzyme activity was determined from the oxidation of reduced NADPH in the presence of H_2O_2 used as substrate. The decrease in concentration of NADPH was monitored and recorded at 340 nm in a mixture containing reduced glutathione and

glutathione reductase. Enzymes units were defined as the number of micromoles of NADPH oxidized per minute. Results were defined as U/ml.

Assay of SOD activity

SOD activity was determined by Cayman's superoxide dismutase assay kit that utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine.²³ The results are presented in U/ml.

Assay of total thiol levels

A spectrophotometric assay based on 2,2-dithiobisnitrobenzoic acid (DTNB or Elman's reagent) is used for total thiol assay.²⁴ An aliquot of serum is mixed with Tris-EDTA buffer, then DTNB is added. After 15 min incubation at room temperature, the absorbance is measured at 405 nm. A reagent blank without sample and a sample blank with methanol instead of DTNB were prepared in a similar manner. GSH (50-100 $\mu\text{mol/l}$) solution is used as calibrator. Total thiol levels were expressed as $\mu\text{mol/l}$.

Statistical analysis

SPSS 15.0 (Statistical Packages for Social Sciences; SPSS Inc., Chicago, Illinois, USA) program was used for statistical analysis of data. Kolmogorov-Smirnov test was used to reveal the distribution pattern of data. Parametric and non-parametric tests were used if the data meet the requirements of the tests. Data were expressed as mean SE in parametric test and median and inter-quartile range (IQR) in non-parametric test. One Way Anova (Tukey) test and Kruskal Wallis test were used to compare groups. Cut-off values were determined by curve estimation test. A p value of <0.05 was taken to be statistically significant.

RESULTS

Baseline characteristics of the study groups

Clinical and biochemical characteristics of the study groups are presented in Table 1. Serum AST, ALT, total bilirubin, albumin, BUN, creatinine levels and age were significantly higher in HE groups than in control group. Thirty HE patients were followed during the study period. Serum levels of NO and XO were statistically significantly higher in the HE group according to control group ($p < 0.031$, and $p < 0.001$, respectively). Serum total thiol levels were low in patients with HE than the controls ($p < 0.001$). TAC, GSH-Px and SOD levels were not significantly different in patients with HE than the controls ($p > 0.405$, $p > 0.194$, and $p > 0.19$, respectively) (Table 2). Eight patients died at intensive care unit during follow up period. Twenty-two patients recovered clinically (PHE group). Serum total thiol levels were low and serum NO levels were high in PHE group according to control group ($p < 0.001$, $p < 0.001$, respectively) (Table 3). We also

compared the oxidative and anti-oxidative parameters in encephalopathy group (HE) and recovered periods within the PHE group. XO activity was statistically significantly reduced in recovered patients (PHE group) ($p < 0.01$) while serum TOC levels decreased moderately ($p < 0.05$). Variations in XO levels were apparent. TAC, total thiol, superoxide dismutase, glutathione peroxidase and NO levels were not changed significantly (Table 4). Performances of oxidant and anti-oxidant parameters in hepatic encephalopathy TOS, XO, total thiol, NO

parameters were analyzed by ROC method in HE and control group (Table 5). All of the above-mentioned parameters were significantly different in both groups especially XO and total thiol. XO differed from other parameters with its sensitivity, specificity, positive and predictive values. TOC and XO levels normalized in PHE group however, total thiol and NO levels were still statistically significantly different according to control group. The interesting result was apparent decrease in XO levels in PHE group compared to HE period (Table 6).

Table 1: Clinical and biochemical characteristics of the study group.

Variables	Mean±SD (%)	HE group, n=30 (%)	Control group, n=31 (%)	P value
Male		19 (63)	17 (55)	NS
Female		11 (37)	14 (45)	NS
Age (years)		63±11.9	51±14.6	0.001
Child-Pugh class				
A		0		
B		6 (20)		
C		24 (80)		
Ascitis		20 (67)		
HE grades				
1		4 (13.3)		
2		13 (43.3)		
3		9 (30)		
4		4 (13.3)		
BUN (mg/dl)	25-75	30 (18-50)	14 (12-16)	<0.001
Creatinine (mg/dl)	25-75	1.3 (0.9-2.3)	1 (0.8-1.1)	0.005
AST (U/l)	25-75	64 (38-96)	21 (19-23)	<0.001
ALT (U/l)	25-75	35 (21-82)	19 (15-23)	<0.001
Total bilirubin (mg/dl)	25-75	3.4 (1.9-5.9)	0.4 (0.4-0.5)	<0.001
Albumin (g/dl)	25-75	2.35±0.56	3.83±0.34	<0.001

Table 2: Serum oxidant and anti-oxidant levels in HE and control groups.

Variables	HE group (n=30)	Control group (n=31)	P value
TAC (mean±SD) (µmol/l)	1.26±0.47	1.17±0.38	0.405
TOC (25-75%) (µmol/l)	5.6 (3-9.5)	3.6 (2.3-6.4)	0.043
NO (25-75%) (µmol/l)	17.6 (11.6-23.8)	13.5 (9.9-16)	0.031
GSH-Px (25-75%) (U/ml)	72.7 (53-92)	80 (62-96)	0.194
SOD (mean±SD) (U/ml)	12.3±4.51	13.8±3.98	0.19
Total thiol (25-75) (µmol/l)	239 (194-314)	338 (298-362)	<0.001
XO (mean±SD) (U/ml)	3.08±0.61	1.61±0.55	<0.001

Table 3: Serum oxidant and anti-oxidant levels in PHE group and control groups.

Variables	PHE group (n=22)	Control group (n=31)	P value
TAC (mean±SD) (µmol/l)	1.29±0.39	1.17±0.38	0.283
TOC (25-75%) (µmol/l)	3(2.1-5.2)	3.6(2.3-6.4)	0.437
NO (25-75%) (µmol/l)	20.3 (14-31)	13.5 (9.9-16)	<0.001
GSH-Px (25-75%) (U/ml)	65 (48-88)	80 (62-96)	0.134
SOD (mean±SD) (U/ml)	14.5±7.17	13.8±3.98	0.65
Total thiol (25-75) (µmol/l)	240±74	338±39.7	<0.001
XO (mean±SD) (U/ml)	1.49 (1.1-1.9)	1.61 (1.2-2.1)	0.588

Table 4: Comparisons of serum oxidant and anti-oxidant levels within HE group patients (non-exitus) in hepatic encephalopathy versus recovered periods (PHE) (before treatment versus post treatment).

Variables	HE group (non-ex) (n=22)	PHE group (recovered period) (n=22)	P value
TAC (mean±SD) (µmol/l)	1.26±0.37	1.29±0.39	0.764
TOC (25-75%) (µmol/l)	5.68 (3-9.5)	3 (2.1-5.2)	0.015
NO (25-75%) (µmol/l)	19 (11.6-34.5)	20.3 (14.9-31.8)	0.405
GSH-Px (25-75%) (U/ml)	74.6±27.2	71.6±27.9	0.718
SOD (mean±SD) (U/ml)	10.7±2.97	14.5±7.17	0.12
Total thiol (25-75) (µmol/l)	240 (200-314)	240 (213-301)	0.879
XO (mean±SD) (U/ml)	3.09 (2.6-3.4)	1.49 (1.1-1.9)	0.001

Table 5: To identify sensitivity-specificity and positive (PPV)- negative predictive values (NPV) of parameters HE from control group.

Variables	AUC	Cutt-off	Sensitivity (%)	Specificity (%)	P (area=0.5)	+PV	-PV
TOC (µmol/l)	0.651	4.47	56.7	74.2	0.0343	68.0	63.9
Total thiol (µmol/l)	0.782	277.44	70.0	90.3	<0.0001	87.5	75.7
XO (U/ml)	0.975	2.29	90.0	96.8	<0.0001	96.4	90.9
NO (µmol/l)	0.661	16.01	60.0	77.4	0.0271	72.0	66.7

Table 6: To predict sensitivity-specificity and positive- negative predictive values of parameters in PHE from control groups.

Variables	AUC	Cutt-off	Sensitivity (%)	Specificity (%)	P (area=0.5)	+PV	-PV
TOC (µmol/l)	0.563	2.27	40.9	77.4	0.4510	56.3	64.9
Total thiol (µmol/l)	0.822	265.24	63.64	93.55	<0.0001	87.5	78.4
XO (U/ml)	0.543	1.75	72.7	48.4	0.6035	50.0	71.4
NO (µmol/l)	0.789	16.49	68.18	80.65	<0.0001	71.4	78.1

DISCUSSION

HE is one of the dangerous complications of cirrhosis. Liver dysfunction led increased ammonia concentration in the brain. Hyperammonemia causes a serious nervous system disorder called hepatic encephalopathy. Many mechanisms are under investigation to reveal the exact mechanism of HE. Reactive oxygen species appear to play a crucial role in the pathogenesis of hepatic encephalopathy.²⁵

Serum concentrations of different oxidant species can be measured in laboratories separately, however, as the measurement of different oxidant molecules separately is not practical, and their oxidant effects are additive, measuring TOC of a sample can provide a new and practical approach.²⁶ In our study, serum TOC levels, of which the main components are hydrogen peroxide and lipid hydroperoxide, was increased in patients with HE than those of controls.

This result clearly indicates the presence of increased oxidative stress. Total thiol is known as a strong antioxidant and neutralizes ROS production. Total thiol levels were low both in HE and PHE group in compared to control group. Decreased total thiol levels could possibly be in response to the continuous production of ROS (protein oxidation), which need thiol for detoxification.²⁷

Total thiol groups are very susceptible to oxidation and total thiol groups of proteins are mainly responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress.^{28,29} When the organism is exposed to oxidative stress, total thiol groups are among the first antioxidants that are consumed.³⁰

Thiol levels decreases in cirrhotic patients.²⁹ Thiol levels were still low in-patient population after treatment of HE. For that reasons we interpreted the decreased thiol levels as a consequence of underlying advanced liver disease. The increased TOC levels in HE group may be consequence of either increased oxidative stress or consumption of thiol groups since HE patients have advanced liver disease.²⁹ TAC, which is also referred as total antioxidant activity, was measured. A new-generation, more stable, coloured 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS) was employed for the measurement of TAC.²¹ In our study, serum TAC was not different in patients with HE than those of controls. Even though serum thiol levels were low in HE groups, serum levels of TAC were similar in both HE and control group. This paradox may be explained by significant differences between serum total bilirubin levels, which is one of the components of serum TAC level. Enzymatic compounds are primarily responsible for intracellular defense. In our study, GSH-Px, and SOD levels were similar in HE and control group.

SOD is the first line of defense mechanism against ROS in cells. First, SOD catalyzes the dismutation of O₂⁻ to hydrogen peroxide (H₂O₂). Then GSH-Px converts H₂O₂ to H₂O.²¹ Oxidative stress can be overcome by reducing generation of ROS or increasing the amounts of antioxidants.⁴

Present data revealed that antioxidant enzymes, SOD and GSH-Px, are not changing in HE group. XO levels were significantly high in HE group. XO is an enzyme and classified as a protein. The major sources of XO are liver and intestine, in addition, XO is present in kidney, lungs, myocardium, brain, plasma and erythrocytes. XO is released into the circulation from organ rich in this enzyme following periods of metabolic stress and attack different organ simultaneously by means of oxygen radicals being produced. Both XO from endothelial cells in an organ and circulating XO, therefore, may contribute to the production of free radicals that damage these organs.³¹

Tissue destruction and degeneration can result in increased oxidative damage. Suggested sources of free radicals have been identified which include catecholamine degradation by monoamine oxidase, mitochondrial respiration (distribution of mitochondrial electron transport chains), invading phagocytes, membrane-bound NAD (P) H oxidases, transition metal ion release (iron and copper), arachidonate-metabolising enzymes, and xanthine oxidase.^{32,33} Increased metabolic stress, any more impairment in the circulation of cirrhotic liver and/or systemic circulation due to HE may explain the increased plasma levels of XO in HE patients since XO levels return to normal levels by healing of HE. NO levels were high both in HE and PHE groups compared to control group. That result is expected since studies of experimental portal hypertension have demonstrated that splanchnic vascular endothelial cells are primarily responsible for mediating splanchnic vasodilatation and enhanced portal venous inflow through excess generation of NO.³⁴ NO levels were similar in HE and PHE groups. That result showed that presence of HE did not alter NO generation.

CONCLUSION

In conclusion, serum oxidative stress parameters were increasing in hepatic encephalopathy patients versus recovered however serum anti-oxidant enzymes parameters were not changed. Interestingly, serum XO level was significantly decreased in patients that recovered from hepatic encephalopathy. As a result oxidative system, in systemic circulation, is activated during hepatic encephalopathy and changes in XO level during and after hepatic encephalopathy is very important. This parameter may be a potential marker in differential diagnosis of hepatic encephalopathy and other coma causes.

Therefore, we think that, effective antioxidant therapy to inhibit total oxidation, XO and NO levels and also to increase thiol levels, which are an antioxidant, may be a contributive therapeutic option in the patient with HE.

The limitations of the study are the small number of patients. Supportive studies with more patients and more research are needed.

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Conflict of interest: None declared

Ethical approval: The study was approved by the institutional ethics committee

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