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THE RELEVANCE OF OXIDATIVE STRESS IN CIRRHOTIC PATIENTS WITH DIFFERENT FORMS OF HEPATIC ENCEPHALOPATHY

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Abstract - While the exact ammonia-induced pathogenic mechanisms in hepatic encephalopathy (HE) are not completely understood, an increasing number of reports have lately been involved in studying the possible relevance of oxidative stress status in HE. Here we evaluated the levels of some oxidative stress markers (two main antioxidant enzymes: superoxide dismutase-SOD and glutathione peroxidase-GPX, as well as a lipid peroxidation marker: malondialdehyde-MDA) in thirty-six cirrhotic patients with overt HE (n=12) or minimal HE (n=24), as compared to a control age-matched group (n=19). We also included a group with cirrhosis without HE (n=42). Our results provide additional evidences of increased oxidative stress in both overt and minimal HE, as expressed by altered serum glutathione peroxidase antioxidant activity and increased levels of lipid peroxidation. Moreover, we demonstrated a significant correlation between the levels of the aforementioned oxidative stress markers and the severity of cirrhosis according to the Child and MELD scales, as well as with the venous blood ammonia level.

Key words: Hepatic encephalopathy, oxidative stress

INTRODUCTION

While it is generally accepted that ammonia plays a central role in the pathogenic aspects involved in hepatic encephalopathy (HE) (Hazell et al., 1999), the exact mechanisms of its neurotoxicity remain poorly understood.

Oxidative stress, which is the condition arising from an imbalance between toxic reactive oxygen species (ROS) and antioxidant systems (Sies, 1997), has been reported to be implicated in the development and the progression of various pathological conditions (Halliwell and Gutteridge, 2007), including acute and chronic liver diseases. In this way, a previous number of studies have focused on the role of oxidative stress in liver injury (Chojkier et al., 1989, Yasa et al., 1999, Chen et al., 1997, Bhandari et al., 2008, Cesaratto et al., 2004).

Additionally, an increasing number of studies in various animal models (Kosenko et al., 1997, 1999, 2003, Norenberg et al., 1992, Robb et al., 1998) have described a possible relevance of oxidative stress as a pathogenetic mechanism involved in HE (Bosoi et al., 2012). Still, there are very few studies regarding these aspects in human patients, only related to alcohol poisoning-induced oxidative stress status in HE (Negru et al., 1999).

The aim of the present study was to evaluate the levels of some oxidative stress markers (two main

antioxidant enzymes: superoxide dismutase-SOD and glutathione peroxidase-GPX, as well as a lipid peroxidation marker: malondialdehyde-MDA) in patients with both overt HE and minimal HE, as compared to an age-matched group, but also including a group with cirrhosis without any form of HE. Moreover, we were interested in establishing the correlations between the levels of the aforementioned oxidative stress markers and the results of the Child and MELD specific scales, as well as with the venous ammonia level.

METHODS

Subjects

The prospective study included 78 patients aged between 41 and 75 years (average 56.3 ± 1.53 years), of which 52 were males and 26 females, with a definite diagnosis of cirrhosis, in a stable condition, recruited from the outpatient clinic or hospitalized between January 2012 and August 2012 in the Gastroenterology Department of the Universitary St. Spiridon Hospital, Iasi, Romania.

The study was conducted according to the provisions of the Declaration of Helsinki and was approved by the local ethics committee. The protocol was explained to at least one relative of each subject and written informed consent was obtained from all selected patients or their relatives.

Exclusion criteria were diagnosis of a neurological or psychiatric disease, current use of psychotropic medications, co-existing diseases like acute infections, diabetes mellitus or systemic uncontrolled disease (cardiac, respiratory, kidney failure), gastrointestinal bleed or blood transfusion within previous 2 weeks, smoking and recent (< 6 months) active alcoholism, as well as the absence of informed consent. Subjects taking antioxidant supplements were also excluded. The ongoing medication was not suspended before the tests since the suspension of treatment in cirrhotic patients was considered unethical.

The etiology of cirrhosis was alcohol in 34 patients, chronic viral hepatitis in 36 patients, while both alcohol and viral factor were found in 8 cases. Patients had different grades of liver failure according to Child classification (A-C) and MELD score (10-25).

HE was assessed using the West Haven mental status scale and motor disturbances evidences (asterixis). West Haven criteria are defined as follows: grade 0 – no detectable changes in behavior or personality; grade 1 – trivial loss of awareness, euphoria or anxiety, shortened attention span, impaired performance of addition; grade 2 – lethargy or apathy, impaired performance of subtraction, minimal disorientation to time or place, subtle personality change, inappropriate behavior; grade 3 – confusion, gross disorientation, somnolence to semi-stupor (may respond to verbal stimuli); and grade 4 – coma (no response to verbal or noxious stimuli). Patients with grade 0 of HE were tested for minimal HE using the psychometric hepatic encephalopathy score (PHES), a standard battery of neuropsychological tests including number connection tests (NCT) A and B, digit-symbol test (DST), line- tracing test (LTT) and serial-dotting test (SDT). The minimal HE score was determined using an online free calculator, available at http://www.redeh.org/TEST_phes.htm; minimal HE was defined as a score value ≤-5.

Of the 78 patients, 12 were diagnosed with overt HE, 24 had minimal HE and 42 had neither overt HE nor minimal HE.

The control group consisted of 19 healthy subjects recruited from hospital personal and matched to the patients by age and gender (12 males and 7 females, aged 54.2 ± 5.6 years). In this way, the analysis of covariance showed that patients from all three groups did not differ significantly from healthy comparison subjects, with respect to age and gender (Table 1).

Biochemical estimations

Blood samples were collected in the morning, before breakfast, allowed to clot and centrifuged immediately. Serum was aliquoted into Eppendorf tubes and stored at -40°C until measurement.

Table 1. Demographic data in the control, overt HE, minimal HE and non-HE groups.

	Control[a] (n=19)	Overt HE group[a] (n=12)	Minimal HE group[a] (n=24)	Non-HE group[a] (n=42)	F[b]	P[b]
Age (years)	54.2 ± 5.6	58.6 ± 5.3	54.7 ± 4.2	56.1 + 4.4.	0.22	0.803
Gender (male/ female)	12/7	8/4	17/7	27/15	0.14	0.867

[a] Each value represents mean ± standard deviation
[b] Analysis of covariance

Determination of SOD

Superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of the enzyme with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD Assay Kit (FLUKA, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide anions) after 20 min of reaction time at 37°C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

Determination of GPX

The glutathione peroxidase (GPX) activity was measured using the GPX cellular activity assay kit CGP-1 (SIGMA). This kit uses an indirect method based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled with recycling GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH at 340 nm during oxidation of NADPH to NADP is indicative of GPX activity.

Determination of MDA

Malondialdehyde levels were determined by thiobarbituric acid reactive substances (TBARs) assay. 200 µL of serum were added and briefly mixed with 1 mL of trichloroacetic acid at 50%, 0.9 mL of TRIS-HCl (pH 7.4) and 1 mL of thiobarbituric acid 0.73%. After vortex mixing, samples were maintained at 100°C for 20 min; the samples were then centrifuged at 3000 rpm for 10 min and the supernatant read at 532 nm. The signal was read against an MDA standard curve, and the results were expressed as nmol/ml (Padurariu et al., 2010a).

Data Analysis

The levels of oxidative stress markers (SOD, GPX and MDA) were statistically analyzed by using one-way analysis of variance (ANOVA). All results are expressed as mean ± SEM. *Post hoc* analysis were performed using Tukey's honestly significant difference test in order to compare groups. F values for which $p<0.05$ were regarded as statistically significant. Pearson's correlation coefficient was used to investigate the possible correlations between the aforementioned oxidative stress markers and the results of the Child and MELD specific scales, as well as with the venous ammonia level.

RESULTS

Regarding the specific activity of SOD, first of all we observed a significant group difference ($F(3.93)=11$, $p<0.0001$) (Fig. 1). *Post-hoc* comparisons also showed a significant increase in SOD activity in overt HE group ($p<0.0001$) vs. controls, as well as in the minimal HE group ($p<0.0001$) and non-HE group ($p<0.0001$), when compared to control patients. On

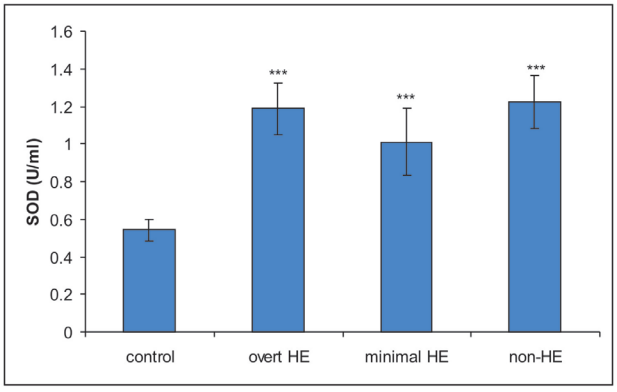

Fig. 1. Superoxide dismutase specific activity in the serum of control, overt HE, minimal HE and non-HE groups. The values are mean ± SEM. (n = 19 in control, 12 in overt HE, 24 in minimal HE and 42 in non-HE group). ***p < 0.0001 vs. control group.

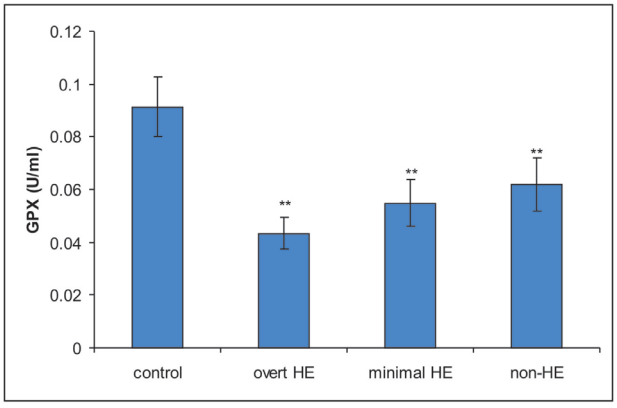

Fig. 2. Glutathione peroxidase specific activity in the serum of control, overt HE, minimal HE and non-HE group. The values are mean ± SEM. (n = 19 in control, 12 in overt HE, 24 in minimal HE and 42 in non-HE group). **p < 0.003 vs. control group.

the other hand, no significant differences were found between overt HE vs. minimal HE (p=0.303), HE vs. non-HE (p=0.818) and minimal HE vs. non-HE groups (p=0.088) (Fig. 1).

Concerning GPX, we also noted significant differences between our groups (F(3.93)=7, p=0.00013) (Fig. 2). Moreover, when we performed the *post-hoc* analysis, we observed a significant decrease in GPX specific activity in overt HE (p=0.003), minimal HE (p=0.002) and the non-HE group (p=0.003), as

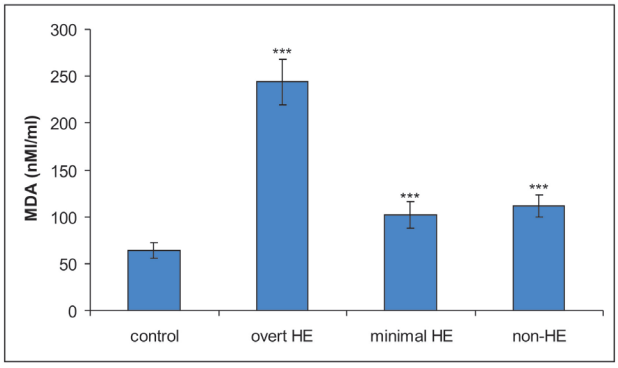

Fig. 3. Malondialdehyde concentration in the serum of control, overt HE, minimal HE and non-HE group. The values are mean ± SEM. (n = 19 in control, 12 in overt HE, 24 in minimal HE and 42 in non-HE group). ***p < 0.0001 vs. control group.

compared to the controls. In addition, a significant decrease of GPX was found in the overt HE group, as compared to the non-HE group (p=0.027), but no significant differences between overt HE vs. minimal HE (p=0.161) and minimal HE vs. non-HE groups (p=0.274) (Fig. 2) were found.

As mentioned, our analysis also included a peroxidation marker (MDA). In this case we observed very significant differences between our groups (F(3.93)=47, p<0.0001) (Fig. 3). Additionally, *post-hoc* analysis showed a significant increase of MDA in the overt HE group (p<0.0001), minimal HE group (p<0.0001), as well as in the non-HE group (p<0.0001) when compared to the controls. A significant increase in MDA concentration was also seen in the overt HE group, as compared to the minimal HE group (p<0.0001) or non-HE group (p<0.0001). However, no significant differences were observed between minimal HE and non-HE group (p=0.308) (Fig. 3).

When we analyzed the correlations between the previously described oxidative stress markers and the levels of venous ammonia, we observed significant correlations between GPX vs. venous blood ammonia (n=78, r= -0.233, p= 0.043) and for MDA vs. venous blood ammonia (n=78, r= 0.519, p< 0.0001), but not for SOD vs. venous blood ammonia (n=78, r= -0.045, p= 0.696).

Similarly we found significant correlations between the oxidative stress markers and MELD and Child scales for GPX: GPX vs. MELD (n=78, r= -0.331, p= 0.003)/GPX vs. Child (n=78, r= -0.230, p= 0.048) and MDA: MDA vs. MELD (n=78, r= 0.428, p< 0.0001)/MDA vs. Child (n=78, r= 0.324, p= 0.004), but not for SOD: SOD vs. MELD (n=78, r= -0.061, p= 0.593)/SOD vs. Child (n=78, r= -0.173, p= 0.13).

DISCUSSION

The results described here provide additional evidence of increased oxidative stress in overt HE and minimal HE, as expressed by altered serum glutathione peroxidase antioxidant activity and increased levels of lipid peroxidation. Moreover, we demonstrated a significant correlation between the levels of the aforementioned oxidative stress markers and the results of Child and MELD specific scales, as well as with the venous blood ammonia level.

While the exact ammonia-induced pathogenic mechanism in HE is still not completely understood, recently an increasing number of authors have been studying the relevance of the oxidative stress status in HE. In this way, it was mainly demonstrated that oxidative stress seems to be implicated in the pathogenesis of HE and hyperammonemia (Noremberg, 2003, Rama et al., 2012, Schliess et al., 2006), as demonstrated by decreased levels of antioxidants (SOD, GPX and catalase) (Kosenko et al., 1997, 1999, Bosoi et al., 2012, Geetha et al., 2007), as well as by the increased concentrations of ROS, expressed as increased levels of hydroxyl radicals, superoxide or lipid peroxidation processes (Kosenko et al., 2003, Negru et al., 1999, Hilgier et al., 2003, Norenberg et al., 2004).

In our study, we demonstrated a decreased activity of GPX and increased serum levels of MDA, as a lipid peroxidation marker, but also an increased specific activity of SOD.

This could be explained by the fact that SOD is the first line of defense against oxidative stress development. Therefore, this increase could represent a compensatory process as a result of elevated superoxides levels, which were previously demonstrated, for example, at the mitochondrial level in ammonia-exposed rats (Kosenko et al., 1997, Norenberg et al., 2004).

In the same way, the decrease in specific activity of GPX could be explained by the low-levels of its substrate GSH (glutathione), which was previously reported to be decreased in patients with HE (Norenberg et al., 2004).

In a very recent study published in 2012 by Dhanda, it was demonstrated that a rat bile duct ligation-induced model of HE would result in a significant increase in central SOD specific activity, which is attenuated by the administration of N-acetyl-L-cysteine (Dhanda et al., 2012).

The increased activity of SOD, which is a response to an increase in the production of ROS, leads to increased amounts of hydrogen peroxide that is then degraded by GPX in its low concentrations. Since GPX activity is dependent on its substrate GSH, this antioxidant is rapidly consumed in oxidative stress processes, since GPX could not function appropriately in low GSH concentrations (Sies, 1997).

The explanation for this involves the fact that superoxide dismutase (SOD) and glutathione peroxidase (GPX) are critical antioxidant enzymes that act cooperatively at different sites in the metabolic pathway of free radicals, and an altered activity of one of the enzymes without compensatory changes in the other enzymes may result in increased oxidative stress. In this way, the superoxide radical, spontaneously or with the catalyses of SOD, is converted to hydrogen peroxide, which is reduced to water and oxygen molecules (Halliwell and Gutteridge, 2007).

The findings of the present study could suggest that SOD activity is increased in response to increased reactive oxygen species production. On the other hand, lack of an accompanying similar increase in GPX activity might be one of the contributors of

the increased lipid peroxidation, thus increasing MDA levels.

The aforementioned results could be extremely important, considering that currently the exact mechanism which results in the generation of increased free radicals in HE is not fully understood (Seyan et al., 2010).

There are a few theories in this area of research such as the "two-hit" one, which was recently proposed by Seyan et al., whereby it is considered that initial liver dysfunctions leading to hyperammonemia will lead to specific astrocyte dysfunctions ("initial hit") and only after that inflammation and oxidative stress are kicking in, leading eventually to neuropsychological deterioration (Seyan et al., 2010). Our group has also previously demonstrated the relevance of oxidative stress in various neuropsychiatric disorders (Padurariu et al., 2010b, Ciobica et al., 2010, 2011 a,b,c, 2012, Foyet et al., 2011, Hritcu et al., 2011, Stefanescu and Ciobica 2012, Bild and Ciobica 2012, 2013).

Another important aspect seems to be represented by the disturbed cerebral energy and various mitochondrial injuries (Rama Rao et al., 2012) or manganese toxicity (Norenberg et al., 2004). It was demonstrated that Alzheimer type II astrocytes, which are an important hallmark of HE, are containing peroxidized lipids (Norenberg et al., 2004, Brunk et al., 1999), supporting in this way the relevance of oxidative stress in HE.

Furthermore, our results also confirm that oxidative stress is implicated in the pathogenesis of cirrhosis (Natarajan et al., 2006, Sakena et al., 2010). However, unlike previous studies (Bhandari et al., 2008, Yasa et al., 1999, Geetha et al., 2007, Hanafy et al., 2009), we showed here an increase of SOD specific activity, which could be explained as a compensatory process resulting probably from the elevated ROS levels.

Of course, all these aspects demonstrating the importance of oxidative stress in HE have led to the hypothesis of using antioxidants as potential treatment. A good example in this area is the usage of N-acetyl cysteine, which is known to be an antioxidant that contains a donor sulfhydryl group and which is a precursor of glutathione (Dhanda et al., 2012). There are some evidences regarding its utility in fulminant hepatic failure (Norenberg et al., 2004, Jones et al., 1998, Wendon et al., 1994). Additionally, it was very recently demonstrated that it could have protective effects in a rat bile duct ligation-induced model of HE, as expressed through decreased lipid peroxidation and an increase in the activity of antioxidant enzymes, as well as a significant improvement in the activity of liver marker enzymes, restored structural morphology of liver and a significant amelioration of the spatial memory/motor coordination and cortex or cerebellum structural deficits (Dhanda et al., 2012).

There are several limitations to the present study regarding the heterogeneous groups used and the small size of the samples. Ongoing medication was not suspended for ethical reasons and the high risks of complications in cirrhotic patients, but we have focused on ruling out any factor such as active alcoholism or smoking, gastrointestinal bleeding and acute infections, which could influence the determined oxidative stress markers.

Further studies should delve deeper into the mechanisms and connections that might exist between these aspects and the role of the antioxidants in preventing and treating HE.

CONCLUSION

The present study provides additional evidence regarding increased oxidative stress in HE, demonstrated by altered antioxidant activity and increased levels of lipid peroxidation. Additionally, we have demonstrated a significant correlation between the levels of the determined oxidative stress markers and the results of Child and MELD scales, as well as with the venous ammonia levels.

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