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**ABSTRACT**

Patients with minimal hepatic encephalopathy (MHE) show increased oxidative stress in blood. We aimed to assess whether MHE patients show alterations in different types of blood cells in (a) basal reactive oxygen and nitrogen species levels; (b) capacity to metabolise these species. To assess the mechanisms involved in the altered capacity to metabolise these species we also analysed: (c) peroxynitrite formation and (d) peroxynitrite reaction with biological molecules. Levels of reactive oxygen and nitrogen species were measured by flow cytometry in blood cell populations from cirrhotic patients with and without MHE and controls, under basal conditions and after adding generators of superoxide (plumbagin) or nitric oxide (NOR-1) to assess the capacity to eliminate them. Under basal conditions, MHE patients show reduced superoxide and peroxynitrite levels and increased nitric oxide (NO) and nitrotyrosine levels. In patients without MHE plumbagin strongly increases cellular superoxide, moderately peroxynitrite and reduces NO levels. In MHE patients, plumbagin increases slightly superoxide and strongly peroxynitrite levels and affects slightly NO levels. NOR-1 increases NO levels much less in patients with than without MHE. These data show that the mechanisms and the capacity to eliminate cellular superoxide, NO and peroxynitrite are enhanced in MHE patients. Superoxide elimination is enhanced through reaction with NO to form peroxynitrite which, in turn, is eliminated by enhanced reaction with biological molecules, which could contribute to cognitive impairment in MHE. The data show that basal free radical levels do not reflect the oxidative stress status in MHE.

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**Introduction**

Cirrhotic patients may develop minimal hepatic encephalopathy (MHE) with mild cognitive impairment, attention deficits, psychomotor slowing and reduced visuomotor and bimanual coordination which are not evident but may be unveiled using psychometric tests [1–3]. MHE reduces the quality of life and lifespan of the patients and increases their risk of accidents, falls and hospitalisations. MHE affects more than 4 million people in the USA and Europe and is an important health, social and economic problem [4,5].

Hyperammonaemia and inflammation act synergistically to induce the neurological alterations associated with MHE [6–8]. The underlying mechanisms are not known in detail. Both hyperammonaemia and inflammation may induce oxidative stress, which could contribute to the neurological alterations in MHE. Oxidative stress has been reported in the blood of patients with liver disease [9–12]. Patients with MHE show increased oxidative stress in blood compared to cirrhotic patients without MHE, with increased lipid peroxidation, DNA oxidation, protein carbonylation, 3-nitrotyrosine and oxidized glutathione/reduced glutathione ratio (GSSG/GSH) [13].

The increase of markers of oxidative stress in blood of cirrhotic patients occurs in spite of enhanced...
activities of antioxidant enzymes in erythrocytes and mononuclear cells compared to control subjects [13]. This suggests that both the generation and the detoxification of reactive oxygen and nitrogen species would be increased in cirrhotic patients. However, it is not known whether these increases are different in patients with and without MHE.

Serum levels of 3-nitrotyrosine, a marker of oxidative stress [14], are increased in cirrhotic patients with MHE but not in patients without MHE and these levels are a good biomarker for the diagnosis of MHE [15]. 3-nitrotyrosine is formed by reaction of tyrosine with peroxynitrite [16], which is formed by the reaction of nitric oxide (NO) with superoxide [17,18].

We proposed the hypothesis that increased peroxynitrite formation would mediate the synergistic effects of hyperammonaemia and inflammation in inducing cognitive and motor impairment in MHE [13]. This would imply that the formation of peroxynitrite and its reaction with biological molecules should be increased in patients with MHE compared to patients without MHE and controls without liver disease. The main aim of this work was to assess if this is actually occurring.

To analyse reactive oxygen species and peroxynitrite formation under conditions similar to in vivo, we performed the studies in intact mononuclear peripheral blood cells freshly isolated from cirrhotic patients without MHE or with MHE and from control subjects. To get a wide view of the effects in different cell types, we analysed separately, by flow cytometry, the effects in the following cell populations and subpopulations: leukocytes, lymphocytes, B lymphocytes, T lymphocytes, T CD8⁺ lymphocytes, T CD4⁺ lymphocytes.

The specific aims of this work were to assess whether there are differences in patients with MHE compared to patients without MHE or controls in a) basal levels of main reactive oxygen and nitrogen species, b) capacity to metabolise these species, c) formation of peroxynitrite, and d) reaction of peroxynitrite with biological molecules.

To reach these aims, we measured basal levels of the main reactive oxygen and nitrogen species: mitochondrial and cellular superoxide, peroxynitrite, NO and reduced thiol levels. To assess the capacity to metabolise these species, we analysed the changes in response to an acute stressor with a superoxide-generating agent (plumbagin, Plbg). We also analysed the formation of peroxynitrite by measuring its levels under basal conditions and after addition of Plbg.

To analyse the formation of derivatives of the reaction of peroxynitrite with biological molecules, we measured in serum malondialdehyde for reaction with lipids [19], 8-hydroxydeoxyguanosine for reaction with DNA [20,21] and free 3-nitrotyrosine and nitration of protein-bound tyrosine for the reaction of peroxynitrite with free and protein-bound tyrosine [16].

**Materials and methods**

**Patients and controls**

Fifty patients with liver cirrhosis were consecutively recruited from the outpatient clinics in Hospital Clinico and Hospital Arnau de Vilanova of Valencia, Spain. The diagnosis of cirrhosis was based on clinical, biochemical, and ultrasonographic data. Exclusion criteria were history of overt hepatic encephalopathy, recent alcohol intake (<6 months), infection, recent (<6 weeks) antibiotic use or gastrointestinal bleeding, recent (<6 weeks) use of drugs affecting cognitive function, hepatocellular carcinoma, or neurological or psychiatric disorder. Nineteen healthy volunteers were also enrolled in the study once the liver disease was discarded by clinical, analytical, and serologic tests. All participants were included in the study after signing a written informed consent. Study protocols were approved by the Scientific and Ethical Committees of both hospitals. The procedures followed were in accordance with the ethical guidelines of the Declaration of Helsinki. After a standard history and physical examination, blood was drawn for biochemical measurements.

Psychometric tests, attention and coordination tests, and blood collection were carried out on the same day. After performing the psychometric tests, patients were classified as without MHE or with MHE (see section “Neuropsychological Assessment”). Therefore, the study includes three groups: (a) control subjects, (b) patients without MHE, and (c) patients with MHE.

**Neuropsychological assessment**

**Diagnosis of MHE**

MHE was diagnosed using the Psychometric Hepatic Encephalopathy Score (PHES) [1,2]. PHES comprises five psychometric tests: digit symbol test evaluates processing speed and working memory, number connection tests A and B mental processing speed and attention; serial dotting test and line tracing test visuospatial coordination. The scores were adjusted for age and education level using Spanish normality tables (www.redeh.org). Patients were classified as having MHE when the score was ≤−4 points [1,2].

Characterisation of oxidative and nitrosative stress by flow cytometry in blood cell populations.
**Determination of the basal levels of reactive oxygen and nitrogen species**

Venous blood samples (2.5 ml) were taken in BD Vacutainer™ EDTA tubes. Three hundred microliters of whole blood was incubated with 30 μl of a mixture of monoclonal antibodies specific for the different leukocyte subpopulations (see below). Samples were incubated in the dark for 20 min at room temperature, then diluted 1:20 with Phosphate-buffered saline (PBS) and dispensed into propylene tubes in 0.5-ml aliquots. Samples were then incubated for 30 min at 37°C with appropriate fluorescent probes to determine different reactive oxygen and nitrogen species (see below) under basal conditions.

**Analysis of the capacity to eliminate reactive oxygen and nitrogen species**

To analyse the capacity to eliminate superoxide and NO, an additional 0.5-ml aliquot of the same samples used above to analyse basal levels of reactive oxygen and nitrogen species were incubated with plumbagin (Plbg, 10-μg/ml final concentration) (Sigma-Aldrich, St. Louis, MO) as a generator of superoxide anion [22] or with NOR-1 (250 μM, Santa Cruz Biotechnologies, Heidelberg, Germany) as a NO donor [23,24], for 10 min at 37°C. Samples were then incubated for 30 min at 37°C with appropriate fluorescent probes to determine the accumulation of different reactive oxygen and nitrogen species (see below) induced by the exogenously generated superoxide or NO.

The artificial systems to generate superoxide and NO were used to allow analysing the capacity of the cell to eliminate them. These systems generate rapidly large amounts of superoxide and NO which would accumulate if they are not eliminated by the cell. The analysis of superoxide and NO levels after incubation with the artificial generators provides information on the capacity of the cell to eliminate them. Moreover, the analysis of compounds such as peroxynitrite or nitrosylation allows analysing how the cell eliminates these systems generate rapidly the products of the reaction between hydrogen peroxide and NO which would accumulate if they are not eliminated by the cell. The analysis of superoxide and NO levels after incubation with the artificial generators provides information on the capacity of the cell to eliminate them. Moreover, the analysis of compounds such as peroxynitrite or nitrosylation allows analysing how the cell eliminates these systems generate rapidly the products of the reaction between hydrogen peroxide and NO which would accumulate if they are not eliminated by the cell.

**Monoclonal antibodies used**

CD45-Krome Orange clone J.33 (CD45-KO), CD16 APC-AlexaFluor® 750 (clone 3G8), CD19 APC-AlexaFluor700 (clone J3.119), CD4 ECD (Clone SFC12T4D11 (T4)), and CD8 PC7 (clone SFC121Thy2D3 (T8)) were from Beckman Coulter. CD14-Pacific Blue™ (clone M5E2) (CD14-PB), and CD3-APC (clone UCHT1) were from BioLegend (San Diego, CA).

**Fluorescent probes and fluorogenic substrates used**

The fluorescent probes 4-Amino-5-Methylamino-2',7'-Difluoro-Fluorescein diacetate (DAF), dihydrorhodamine 123 (DHR123), 5-chloromethyl fluorescein diacetate (CMF) and MitoSOX™ Red (MTX) were from Molecular Probes. Dihydroethidium (HE), 2',7'-dichlorodihydrofluorescein diacetate (DCF) was from Sigma-Aldrich.

DAF is a cell-permeable fluorogenic substrate which is used to detect and quantify intracellular NO [25].

DHR123 is an uncharged and nonfluorescent substrate indicator that diffuses passively across membranes where it is oxidised to cationic rhodamine 123 and exhibiting green fluorescence. DHR123 is oxidised by peroxynitrite indirectly via the decomposition (radical) products: hydroxyl (•OH), carbonate (O₂•⁻), and nitrogen dioxide (NO₂•) radicals, but not by NO [26–28]. DHR123 can also be oxidised by hypochlorous acid, which is found almost exclusively in neutrophils [26, 27].

MTX is a fluorogenic probe specifically targeted to mitochondria and it is oxidised by superoxide to a fluorescent product with orange fluorescence emission [29].

HE is a cell-permeant redox probe which is selectively oxidised by superoxide to a specific product, 2-OH-ethidium, emitting orange fluorescence when bound to DNA [30]. DCF is readily oxidised to a fluorescent molecule, 2',7'-dichlorofluorescein, in the presence of products of the reaction between hydrogen peroxide and various cellular peroxidases, as well as radical oxidants (including •OH, O₂•⁻, and NO₂•) and thiolderived radicals [28, 31, 32].

CMF is a fluorescent indicator of intracellular thiol status, including reduced glutathione [33, 34]. The final assay concentration for each fluorochrome was: DAF: 2 μM; DHR123: 2.9 μM; MTX: 1.25 μM; HE: 1.6 μM; DCF: 10.3 μM; CMF: 0.05 μM.

**Determination of tyrosine nitration in serum proteins by Western blot**

Forty or 80 micrograms of serum protein were subjected to immunoblotting as in Felipo et al. [35]. Primary antibody against nitrotyrosine (1:500) was from Hycult. Secondary antibodies were antimouse IgG.
Free levels of 3-nitrotyrosine in serum was measured as in Montoliu et al. [15]. Malondialdehyde and 8-hydroxydeoxyguanosine serum levels were quantified as in Giménez-Garzó et al. [13].

**Statistical analysis**

Values are given as mean ± SEM. Results were analysed by one-way ANOVA followed by post-hoc Bonferroni’s multiple comparison test. Analyses were performed using GraphPad Prism version 6.0 and SPSS version 19.0 (Chicago, IL) and two-sided \( p \) values < .05 were considered significant.

**Results**

We assessed by flow cytometry the fluorescence levels of different probes for measurement of reactive oxygen and nitrogen species in different types of peripheral blood mononuclear cells.

As an indicator of radical formation, in general, we measured the DCF fluorescence. DCF fluorescence was not affected in cirrhotic patients without MHE and was increased in patients with MHE in leukocytes, T lymphocytes and neutrophils (Figure 1, Table S1, Supplementary Figure 1).

Mitochondrial superoxide levels (measured as MTX fluorescence) were increased similarly in cirrhotic patients with or without MHE compared to control subjects in leukocytes, total lymphocytes and B lymphocytes, and in neutrophils but not in T lymphocytes (Figure 2, Table S2, Supplementary Figure 2). Mitochondrial superoxide level was increased selectively in cirrhotic patients with MHE compared to controls or patients without MHE in monocytes (Supplementary Figure 2).

Results of HE fluorescence show that cellular superoxide levels were not affected in cirrhotic patients without MHE and was significantly lower in patients with MHE than in patients without MHE or controls in all cell types analysed (Figure 3, Table S3, Supplementary Figure 3).

NO levels, measured by DAF fluorescence, were increased in cirrhotic patients with or without MHE compared to control subjects in all cell types analysed, except in monocytes and neutrophils. The increase was larger in patients with MHE than without MHE in all types of lymphocytes analysed (Figure 4, Table S4, Supplementary Figure 4).

DHR123 fluorescence was not affected in cirrhotic patients without MHE (except in neutrophils) and was significantly lower in patients with MHE than in patients without MHE or controls in all cell types analysed, except for monocytes and neutrophils (Figure 5, Table S5, Supplementary Figure 5).

Reduced thiol (mainly GSH) levels were increased in cirrhotic patients with or without MHE compared to control subjects in all cell types analysed. The increase in CMF fluorescence tends to be larger in patients with MHE than without MHE in all cell types analysed except in B lymphocytes (Figure 6, Table S6, Supplementary Figure 6).

The above data show changes associated to MHE in the levels of cellular superoxide, NO, peroxynitrite-derived radicals and reduced thiol levels. These changes may be due to alterations in the generation and/or the elimination of free radicals. To evaluate the capacity of elimination of superoxide, we analysed the responses to addition of the superoxide-generating agent plumbagin (Plbg).

Plbg also increased the DCF fluorescence in all cell types. The ratios of DCF fluorescence after/before Plbg were similar in patients without MHE and controls and were higher in patients with MHE, especially in T lymphocytes, leukocytes, monocytes and neutrophils (Figure 1, Table S1, Supplementary Figure 1). This could indicate an increased conversion of superoxide to hydrogen peroxide by superoxide dismutase (SOD) in patients with MHE, although other free radicals could be generating, leading to the increase in DCF fluorescence.

Plbg strongly increased mitochondrial superoxide levels in all cell types in controls and patients (Figure 2, Table S2). To evaluate the capacity to eliminate mitochondrial superoxide, we quantified the ratio of mitochondrial superoxide after and before Plbg addition. The ratios were similar in all cell types in controls and patients except for T lymphocytes and for B lymphocytes, in which the increase was lower in patients than in controls and lower in patients with than without MHE (Figure 2, Table S2, Supplementary Figure 2).

Plbg also increased cellular superoxide levels. The increase was similar in cirrhotic patients without MHE and controls and was strongly reduced in patients with MHE in all cell types except in monocytes (Figure 3, Table S3, Supplementary Figure 3), indicating a higher capacity to eliminate superoxide in these patients.

Plbg induced a strong increase in DHR123 fluorescence, indicating that a relevant part of the
superoxide generated by Plbg could be reacting with NO to form peroxynitrite (Figure 5, Table S5). The increase in DHR123 fluorescence was similar in patients without MHE and in controls and was strongly increased in patients with MHE in all cell types, as reflected in ratios after/before Plbg about two-fold higher than in patients without MHE (Figure 5, Table S5, Supplementary Figure 5). This indicates that larger amounts of superoxide and NO are reacting to form peroxynitrite in patients with MHE.

Plbg induced a slight decrease in NO levels in controls and patients without MHE. The change was smaller in patients with MHE (Figure 7, Table S7). This further supports that part of the superoxide generated by Plbg is reacting with NO to form peroxynitrite.
Plbg reduced the levels of reduced thiols, most likely due to activation of glutathione peroxidase and increased consumption of GSH to eliminate hydrogen peroxide (see Figure 9). The ratios of reduced thiol after/before Plbg were similar for controls and patients without or with MHE in all cell types except in B lymphocytes and monocytes, in which the reduction was lower in patients than in controls (Figure 6, Table S6, Supplementary Figure 6).

To evaluate the capacity of elimination of NO, we analysed its levels before and after addition of the NO-generating agent NOR-1. NOR-1 increased the levels...
of NO similarly in controls and patients without MHE. The increase was lower in patients with MHE, indicating an increased capacity to eliminate NO in patients with MHE (Figure 4 and Table S4).

As indicators of peroxynitrite reaction with biological molecules, we measured the serum levels of markers of reaction with lipids (malondialdehyde), DNA (8-hydroxydeoxyguanosine) and tyrosine (3-nitrotyrosine).

Malondialdehyde levels were increased in cirrhotic patients without MHE (0.5 ± 0.018 nM, \( p < .001 \)) compared with controls (0.21 ± 0.01 nM) and were more strongly increased (0.7 ± 0.03 nM, \( p < .001 \) vs. patients without MHE) in patients with MHE.
8-hydroxydeoxyguanosine levels were not different in controls (9.5 ± 0.4 ng/ml) and cirrhotic patients without MHE (10 ± 0.4 ng/ml) and were significantly increased (12.5 ± 0.7 ng/ml, *p < .05) in patients with MHE.

3-nitro-tyrosine levels were not different in controls (7 ± 0.6 nM) and cirrhotic patients without MHE (9.9 ± 1 nM) and were significantly increased (25 ± 4 nM, *p < .001) in patients with MHE.

Figure 4. Nitric oxide levels under basal conditions and after NOR-1 addition in the different cell populations. Nitric oxide was measured using 4-Amino-5-Methylamino-2',7'-Difluorofluorescein diacetate (DAF) under basal conditions and after addition of NOR-1 in different cell types (left panel of each graph). The ratio of the intensity after and before NOR-1 addition is given in the right panel of each graph. The data are given in Table S4. Values are the mean ± SEM of the following number of individuals: control *n* = 19, without MHE *n* = 22, MHE *n* = 28. Values significantly different from control subjects are indicated by asterisks *.*. Values significantly different in patients with MHE compared to NMHE are indicated by #. Values which are significantly different after NOR-1 than basal levels within each group are indicated by “a”. *#* *p* < .05; **##* *p* < .01; ***###* *p* < .001.
These results indicate an increased reaction of peroxynitrite-derived radicals with free tyrosine to form 3-nitrotyrosine in patients with MHE. To assess whether nitration of tyrosine is also increased in proteins, we analysed by western blot nitration of tyrosine in serum proteins. The blots revealed two main proteins with Mr 25 and 50 kDa, respectively, showing tyrosine nitration. Nitration of tyrosine restudies in the 50 kDa band was significantly increased in patients with MHE (188 ± 14% of controls) compared to patients without MHE (131 ± 19% of controls) or controls (Figure 8(A)). Nitration of tyrosine restudies in the 25 kDa band was

Figure 5. Levels of peroxynitrite-derived species under basal conditions and after plumbagin (Plbg) addition in the different cell populations. These levels were measured using DHR under basal conditions and after addition of plumbagin (Plbg) in different cell types (left panel of each graph). The ratio of the intensity after and before Plbg addition is given in the right panel of each graph. The data are given in Table S5. Values are the mean ± SEM of the following number of individuals: control \( n = 19 \), without MHE \( n = 22 \), MHE \( n = 28 \). Values significantly different from control subjects are indicated by asterisks \(^*\). Values significantly different in patients with MHE compared to NMHE are indicated by \#. Values which are significantly different after Plbg than basal levels within each group are indicated by \( \alpha \). \(* , \# , \# \# \ p < .05 ; \* * , \# \# \# \ p < .01 ; \* * * , \# \# \# \# \ p < .001.\)
Figure 6. Reduced thiol levels under basal conditions and after plumbagin (Plbg) addition in the different cell populations. Reduced thiol levels were measured using 5-chloromethylfluorescein diacetate (CMF) under basal conditions and after addition of plumbagin (Plbg) in different cell types (left panel of each graph). The ratio of the intensity after and before Plbg addition is given in the right panel of each graph. The data are given in Table S6. Values are the mean ± SEM of the following number of individuals: control n = 19, without MHE n = 22, MHE n = 28. Values significantly different from control subjects are indicated by asterisks *. Values significantly different in patients with MHE compared to NMHE are indicated by #. Values which are significantly different after Plbg than basal levels within each group are indicated by “a”. *#, #p < .05; **#, ##p < .01; ###, ###, aap < .001.
Figure 7. Changes in nitric oxide levels after plumbagin (Plbg) addition in the different cell populations. 4-Amino-5-Methylamino-2',7'-Difluorofluorescein diacetate (DAF) fluorescence intensity was measured under basal conditions and after plumbagin (Plbg) addition. The change in the intensity after Plbg addition is given. Values are the mean ± SEM of the following number of individuals: control $n = 9$, without MHE $n = 8$, MHE $n = 4$. Although the differences between groups are not statistically significant, it is clearly seen that nitric oxide decreases following Plbg addition in all groups. The decrease is smaller in patients with MHE.
also increased in patients with MHE (296 ± 69% of controls) compared to patients without MHE (197 ± 39% of controls) or controls (Figure 8(B)).

**Discussion**

Under basal conditions, when the parameters are measured directly in freshly isolated peripheral mononuclear blood cells, patients with MHE show lower levels of superoxide and peroxynitrite-derived radicals and higher levels of NO than patients without MHE and controls. Patients with MHE also show higher levels than patients without MHE for all products of reaction of peroxynitrite with biological molecules: 8-hydroxydeoxyguanosine from reaction with DNA; malondialdehyde from reaction with lipids; free 3-nitrotyrosine from reaction with free tyrosine and protein-bound nitrotyrosine, from reaction with tyrosine in proteins.

The reduced basal levels of superoxide and peroxynitrite are not due to reduced formation, but to enhanced capacity to eliminate them in patients with MHE.

We previously showed [23] that experimental conditions leading to the intracellular generation of peroxynitrite from both NO and superoxide can be followed by monitoring the sequential variations in the fluorescence emission of NO- and superoxide-sensitive fluorescent markers. Based on these results, and having found that MHE patients show an increased serum and protein-bound 3-Ntyr levels, we assumed that DHR123 fluorescence detected would be mainly caused by peroxynitrite production. Therefore, DHR123 can be a good and sensitive indicator of peroxynitrite.

This work provides new advances in the understanding of the mechanisms by which oxidative stress is altered in patients with MHE compared to patients without MHE. A first main finding of the report is that in patients with MHE the mechanisms to eliminate superoxide and NO are enhanced compared to patients without MHE. The results reported show essentially that patients with MHE have increased capacity to eliminate cellular superoxide and peroxynitrite. This is reflected in several facts as discussed below.

First, the addition of the superoxide-generating agent Plbg induces a strong increase in cellular superoxide in controls and patients without MHE, indicating
a low capacity to eliminate the superoxide generated. In contrast, the increase in cellular superoxide was very small in patients with MHE in spite of the continuous delivery from Plbg, indicating a large capacity to eliminate superoxide.

Once identified this enhanced capacity to eliminate superoxide and NO, we assessed the mechanisms involved in this enhanced elimination and identify that superoxide elimination is enhanced through increased reaction with NO to form peroxynitrite which, in turn, is eliminated by enhanced reaction with biological molecules to form, for example, nitrotyrosine.

This enhanced ability to detoxify superoxide occurs in the cytosol but not in mitochondria, as suggested by the similar response of mitochondrial superoxide to Plbg in patients and controls.

The enhanced detoxifying capacity is also reflected in the significantly lower basal levels of cellular superoxide in patients with MHE compared to patients without MHE or controls.

The data reported also provide information on the mechanisms underlying the increased elimination of superoxide in patients with MHE. A relevant mechanism contributing to the elimination of superoxide is its reaction with NO to form peroxynitrite. Although peroxynitrite is toxic (see below), its formation reduces superoxide levels. This mechanism occurs also in controls and patients without MHE but is strongly activated in patients with MHE, as indicated by the around two-fold larger ratio of peroxynitrite after/before Plbg than in patients without MHE or controls.

Superoxide is also detoxified by SOD to form hydrogen peroxide. This mechanism could also be slightly enhanced in patients with MHE compared to patients without MHE or controls as reflected by the larger ratio of DCF fluorescence, after/before addition of Plbg. We have recently shown that SOD activity is increased in cirrhotic patients compared to control subjects, but is not different in cirrhotic patients with or without MHE [13]. This would explain the low difference in basal DCF fluorescence between the two groups of patients. However, given the lack of specificity of DCF, other oxygen and nitrogen species than hydrogen peroxide could also be oxidising the probe [28].

The excess of hydrogen peroxide formed can be in turn eliminated by glutathione peroxidase with consumption of reduced glutathione (Figure 9). This mechanism is also activated in response to the addition of Plbg, as reflected by the decrease in the levels of GSH. We have recently shown that glutathione peroxidase activity is increased in cirrhotic patients compared to control subjects, and slightly higher in patients with than without MHE [13]. However, the decrease in GSH

found now is similar in controls and patients in most cell types, suggesting that glutathione peroxidase would not be the main contributor to the enhanced capacity of MHE patients to eliminate superoxide.

The above data support the idea that patients with MHE adapt to the increased formation of superoxide by enhancing the capacity to eliminate it mainly by forming peroxynitrite by reaction with NO. This adaptive enhancement is observed specifically in patients with MHE but not in patients without MHE, who show levels similar to controls in basal cellular superoxide and in the formation of peroxynitrite following the addition of Plbg.

These data show that direct measurement of the basal levels of individual free radicals is not appropriate to evaluate the oxidative stress status in pathological situations in which adaptive mechanisms as those reported here are occurring. Patients with MHE show lower levels of cellular superoxide and peroxynitrite in lymphocytes in spite of their enhanced formation because they also have enhanced capacity to eliminate superoxide and peroxynitrite. However, the products of these reactions may be main contributors to the cognitive impairment in MHE.

In spite of this enhanced elimination of superoxide by forming peroxynitrite, patients with MHE show reduced basal levels of peroxynitrite in all cell types analysed. This indicates that they have also enhanced capacity to eliminate peroxynitrite. Peroxynitrite may be eliminated by reaction with tyrosine to form nitrotyrosine, with DNA to form 8-hydroxydeoxyguanosine or with lipids to form malondialdehyde. Patients with MHE, but not patients without MHE, show increased serum levels of 3-nitrotyrosine and 8-hydroxydeoxyguanosine compared to controls. Malondialdehyde is increased in patients without MHE but the increase is larger in patients with MHE. This indicates that patients with MHE have also increased capacity to eliminate peroxynitrite by forming 3-nitrotyrosine and 8-hydroxydeoxyguanosine, which are not affected in patients without MHE. These modifications may contribute to the mechanisms leading to cognitive impairment in MHE.

Increased oxidation of nucleic acids may contribute to MHE. Schliess et al. [36] showed that hyperammonemia and hepatic encephalopathy increases RNA oxidation and proposed that RNA oxidation may impair postsynaptic protein synthesis, which is critically involved in learning and memory consolidation. These authors suggest that RNA oxidation may contribute to disturbances of neurotransmitter systems and gene expression and the cognitive deficits observed in hepatic encephalopathy. The results reported here show
that oxidation of nucleic acids is increased in patients with MHE but not in patients without MHE. This suggests that this DNA or RNA oxidation may be involved in the origin of the neurological alterations at the earliest stages of hepatic encephalopathy, which is MHE.

Also, protein nitration may be involved in the beginning of MHE. We show that peroxynitrite reacts not only with free tyrosine but also with tyrosine in proteins, resulting in protein tyrosine nitration. Although we show here increased nitration of serum proteins, a similar increase in nitration could occur in brain proteins involved in cognitive processes. Görg et al. [37] reported increased tyrosine nitration in brain cortex from cirrhotic patients died with overt hepatic encephalopathy, but not in patients without hepatic encephalopathy. It is likely that protein nitration may be also increased in the brain of patients with MHE as we show here for serum proteins.

Nitration by peroxynitrite modifies the function of key proteins modulating glutamatergic neurotransmission [38]. Peroxynitrite-induced nitration inhibits glutamate transporters [39] and glutamine synthetase [40]. Häussinger et al. [41] already suggested that nitration of critical tyrosine residues in glial proteins may play an important role in the pathogenesis of hepatic encephalopathy. Peroxynitrite may also nitrate and alter the function of neuronal proteins including neurofilaments [42] or N-methyl-D-aspartate (NMDA) receptor [43,44]. It has been proposed that peroxynitrite-induced protein nitration contributes to the pathogenesis of Parkinson’s disease [45] or multiple sclerosis [46]. The results reported here show that increased peroxynitrite formation and protein nitration are hallmarks of MHE which are increased selectively in patients with MHE. This suggests that peroxynitrite-induced alterations in neurotransmission may contribute to the cognitive impairment in patients with MHE.

In summary, the data reported show that patients with MHE show lower levels of cellular superoxide and peroxynitrite in lymphocytes in spite of enhanced formation because they also have enhanced capacity to eliminate superoxide, NO and peroxynitrite. This indicates that measurement of basal levels of free radicals does not reflect the oxidative stress status in MHE. We
also identify some mechanisms involved in this enhanced elimination and identify that superoxide elimination is enhanced through increased reaction with NO to form peroxynitrite which, in turn, is eliminated by enhanced reaction with biological molecules to form, for example, nitrotyrosine. Enhanced elimination of superoxide seems to be due to reaction with NO to form peroxynitrite which, in turn, is eliminated by enhanced reaction with lipids, DNA and free and protein-bound tyrosine to form malondialdehyde, 8-hydroxydeoxyguanosine, free 3-nitrotyrosine and protein-bound nitrotyrosine. The products of reaction of peroxynitrite with biological molecules may contribute to cognitive impairment in MHE.

**Disclosure statement**

The authors declare that they have nothing to disclose.

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