Possible Contribution of Brain Oxidative Stress in Experimental Acute Hepatic Encephalopathy in Rats Role of Minocycline and Vitamin E

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Abstract
Encephalopathy and brain edema are serious complications of liver failure and may lead to rapid death of patients. The present study was designed to show the effects of acute hepatic encephalopathy on the oxidative/nitrosative stress markers in the brain and to investigate the modulation of these markers by minocycline and vitamin E to establish the best way of its prevention. 24 male albino rats were divided into 4 groups: Control group, hepatic encephalopathy group, hepatic encephalopathy+minocycline treated group and hepatic encephalopathy+vitamin E treated group. Galactosamine injection resulted in significant increase in serum ammonia and increase in i-NOS gene expression, heme oxygenase-1 gene expression, levels of nitrite/nitrate in the brain and significant increase in brain water. Minocycline or vitamin E injection resulted in partial reversal of measured parameters compared to hepatic encephalopathy group. It can be concluded that galactosamine injection led to hepatic encephalopathy, brain oedema and increase oxidative stress in brain after 24 hours. Minocycline and vitamin E protected liver and brain from galactosamine induced injury 24 hours after injection.

Key Words: Brain edema – Oxidative stress – Liver failure – Nitric oxide – Animals – Galactosamine.

Introduction
ENCEPHALOPATHY and brain edema are serious central nervous system complications of liver failure. Recent studies using molecular probes and antibodies to cell-specific marker proteins have demonstrated the activation of microglial cells in the brain during liver failure [1]. Microglial activation produces superoxide anions [2].

Vitamin E is the most effective chain-breaking antioxidant within the cell membrane. Vitamin E protects critical cellular structures against damage caused by oxygen-free radicals and reactive products of lipid peroxidation. It has been reported that lipid peroxidation was prevented by vitamin E [4].

Material and Methods
Galactosamine and minocycline were obtained from Sigma (St. Louis, MO, USA) in the form of powder and were dissolved in saline. Control rats were injected by the same amount of saline only. Vitamin E was obtained from Sigma (St. Louis, MO, USA) in the form of powder and was dissolved in olive oil. Control rats were injected the same amount of olive oil only.

A total of 24 male albino rats weighing 200-250gm were divided into the following 4 groups: Group I (Control group): Placebo treated in which rats were injected with saline (1ml/kg) and olive oil (1ml/kg) for 2 days then with saline (5ml/kg); Group II (hepatic encephalopathy group): Rats were injected with galactosamine (2.5g/kg IP) dissolved in 5ml saline to induce hepatic encephalopathy [5]; Group III (hepatic encephalopathy+minocycline group): Rats were injected with mi-
necycline (45mg/kg daily IP) dissolved in 1ml saline for 2 days [6] then with galactosamine to induce hepatic encephalopathy after the last dose of minocycline; Group IV (hepatic encephalopathy+ vitamin E group): Rats were injected with vitamin E (150mg/kg daily IP) dissolved in 1ml olive oil for 2 days [7] then with galactosamine to induce hepatic encephalopathy after the last dose of vitamin E. All rats were provided with standard laboratory chow and water and were housed in accordance with institutional animal care policies. The work was done during 2012 in Kasr Al Aini Faculty of Medicine, Cairo University.

Immediately before sacrifice, blood samples were withdrawn 24 hours after galactosamine injection through retro-orbital route using capillary tubes then placed in 10ml eppendorf tubes. The blood samples were used for further determination of ammonia. The animals were sacrificed by cervical dislocation and the brains were excised and sectioned into 2 hemispheres. Tissue samples from the 1st hemisphere were dissected and kept frozen at –80°C in liquid nitrogen until used to measure levels of nitrite/nitrate and gene expression of heme oxygenase-1 and iNOS. The 2nd hemisphere was used to evaluate brain oedema. Brain water was quantitated by the wet-weight/dry-weight method. Half of the brain was weighed before and after 24h incubation in a 1°C oven. Water content of the brain samples was expressed as percentage of water content according to the following equation: \( \% \text{Water} = \frac{(\text{Wet Weight} - \text{Dry Weight})}{\text{Wet Weight}} \times 100 \) [8].

Ammonia was measured in plasma as previously described by Rodrigo [9]. Blood (150 µl) was taken, deproteinized with one volume of ice-cold 6% trichloroacetic acid, and kept on ice for 15min. After centrifugation at 12,000g, for 10min at 4°C, the supernatants were collected, neutralized with 2 M KHCO3, and centrifuged at 12,000g for 10min at 4°C. The neutralized supernatants were used to measure ammonia.

Detection of i-NOS and heme oxygenase-1 gene expression was done by Real time-Polymerase Chain Reaction (real time-PCR).

Determination of brain nitrite/nitrate level based on the reduction of any nitrate to nitrite by a simple metal reduction followed by the detection of total nitrite (intrinsic+nitrite obtained from reduction of nitrate) by Griess reaction.

Data were coded and entered using the statistical package SPSS version 16. Data were summarized using mean, standard deviation and range for the quantitative variable. Comparisons between groups were done using analysis of variance (ANOVA) with multiple comparisons post hoc test in normally distributed quantitative variables while non-parammetrical Kruscal-Wallis Test and Mann-Whitney Test were used for non-normally distributed quantitative variables. Correlations were done to test for linear relations between quantitative variables using Pearson correlation coefficient. Partial correlations were done to control for serum ammonia. \( p \)-values less than 0.05 were considered as statistically significant.

**Results**

Serum ammonia was significantly elevated in hepatic encephalopathy group (Group II) compared to control group (Group I) (94.58 ± 5.8ug/dl versus 12.55 ± 2.48ug/dl) (Fig. 1). The level of i-NOS gene expression showed a significant increase in hepatic encephalopathy group (Group II) compared to control group (Group I) (0.85 ± 0.22 versus 0.04 ± 0.04) (Fig. 2). Furthermore, the levels of heme oxygenase-1 gene expression and nitrite/nitrate showed a significant increase in hepatic encephalopathy group (Group II) compared to control group (Group I) (1.05 ± 0.08 versus 0.02 ± 0.02) and (1.82 ± 0.43 µmolg versus 0.28 ± 0.13 µmolg) respectively (Figs. 3, 4). Brain water showed a significant increase in hepatic encephalopathy group (Group II) compared to control group (Group I) (79.97 ± 1.92% versus 76.34 ± 0.59%) (Fig. 5).

Serum ammonia showed significant decrease in hepatic encephalopathy+minocycline treated group (Group III) compared to hepatic encephalopathy group (Group II) (34.07 ± 10.32ug/dl versus 94.58 ± 5.8ug/dl) but showed significant increase compared to control group (Group I) (34.07 ± 10.32 ug/dl versus 12.55 ± 2.48ug/dl) (Fig. 1). The gene expression of i-NOS showed a significant decrease in hepatic encephalopathy+minocycline group (Group III) compared to hepatic encephalopathy group (Group II) (0.27 ± 0.13 versus 0.85 ± 0.22) but didn’t show significant change compared to control group (Group I) (0.27 ± 0.13 versus 0.04 ± 0.04) (Fig. 2). Furthermore, the gene expression of heme oxygenase-1 showed a significant decrease in hepatic encephalopathy+minocycline group (Group III) compared to hepatic encephalopathy group (Group II) (0.28 ± 0.14 versus 1.05 ± 0.08) but showed significant increase compared to control group (Group I) (0.28 ± 0.14 versus 0.02 ± 0.02) (Fig. 3). Also, the level of nitrite/nitrate showed a significant decrease in hepatic encephalopathy+minocycline group (Group III) compared to hepatic encephalopathy group (Group II) (0.5 ± 0.3 µmolg
Serum ammonia showed significant decrease in hepatic encephalopathy+vitamin E treated group (Group IV) compared to hepatic encephalopathy group (Group II) (36.93 ± 11.79 ug/dl versus 94.58 ± 5.58 ug/dl) but showed significant increase compared to control group (Group I) (36.93 ± 11.79 ug/dl versus 12.55 ± 2.48 ug/dl) (Fig. 1). The gene expression of i-NOS displayed a significant decrease in Hepatic encephalopathy+vitamin E group (Group IV) compared to hepatic encephalopathy group (Group II) (0.42 ± 0.12 versus 0.85 ± 0.22) but showed significant increase compared to control group (Group I) (0.42 ± 0.12 versus 0.04 ± 0.04) (Fig. 2). Furthermore, the gene expression of heme oxygenase-1 showed a significant decrease in hepatic encephalopathy+vitamin E group (Group IV) compared to hepatic encephalopathy group (Group II) (0.48 ± 0.19 versus 1.05 ± 0.08) but showed significant increase compared to control group (Group I) (0.48 ± 0.19 versus 0.02 ± 0.02) (Fig. 3). Also, the level of nitrite/nitrate showed a significant decrease in hepatic encephalopathy+vitamin E group (Group IV) compared to hepatic encephalopathy group (Group II) (74.37 ± 3.61% versus 79.97 ± 1.92%) but didn’t show significant change compared to control group (Group I) (74.37 ± 3.61% versus 76.34 ± 0.59%) (Fig. 5).

No significant change was found between Hepatic encephalopathy+vitamin E group (Group IV) and hepatic encephalopathy+minocycline group (Group III) in gene expression of i-NOS, gene expression of heme oxygenase-1 and the level of nitrite/nitrate in brain and brain water.

There’s strong positive correlation between serum ammonia (ug/dl) and heme oxygenase-1 gene expression (r = 0.868, p = 0.000) and strong positive correlation between serum ammonia (ug/dl) and i-NOS gene expression (r = 0.864, p = 0.000) and strong positive correlation between serum ammonia (ug/dl) and nitrite/nitrate (r = 0.815, p = 0.000) and positive correlation between serum ammonia (ug/dl) and brain water (%) (r = 0.509, p = 0.004).

There’s positive correlation between brain water (%) and heme oxygenase-1 gene expression (r = 0.59, p = 0.001) and positive correlation between brain water (%) and i-NOS gene expression (r = 0.583, p = 0.001) and positive correlation between brain water (%) and nitrite/nitrate (r = 0.533, p = 0.002) however, after controlling of serum ammonia (ug/dl) there is no significant correlation between brain water (%) and i-NOS gene expression, heme oxygenase-1 gene expression and nitrite/nitrate (umol/g) in brain tissues in the four studied groups.
Possible Contribution of Brain Oxidative Stress

Discussion

Hepatic encephalopathy is a serious neuropsychiatric complication of liver failure. Neuronal cell death has been described in end-stage liver failure. However, its prevalence and severity are variable and generally considered to be insufficient to explain the wide range of neuropsychiatric symptoms characteristic of hepatic encephalopathy [10,11].

In the present study, the hepatic encephalopathy group exposed to galactosamine injection showed significant increase in the ammonia levels compared to the control group. These results are in accordance with other studies which stated that serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and ammonia increased 24 hours after injection of galactosamine in rats [5,12]. Sinha et al., [13] mentioned that galactosamine induced liver injury was via the generation of free radicals and depletion of Uridine-5'-triphosphate nucleotides. Also, it was found that apoptotic liver injury that occurred after galactosamine treatment was due primarily to TNF-α release, whereas increased Fas ligand expression appeared to contribute little to the mortality and hepatic injury [14].

In the current study, the hepatic encephalopathy group showed significant increase in heme oxygenase-1 gene expression in the brain compared to the control group. This observation is in agreement with the work of Jiang et al., [3] who reported that hepatic encephalopathy group showed significant increase in heme oxygenase-1 gene expression in the brain compared to control group. In accordance with results of the present work, Jiang et al., [3] reported that heme oxygenase-1 gene expression was elevated in rat with ALF.

Heme oxygenase-1 is induced in response to oxidant stress and converts the oxidant-forming free heme into bilirubin, biliverdin, iron and carbon monoxide. Heme oxygenase-1 (HO-1) can scavenge reactive oxygen species intracellularly via the production of biliverdin, bilirubin and carbon monoxide. Bilirubin has been shown to inhibit NADPH oxidase [15]. In contrast, free iron increases oxidative stress [16]. Although the induction of HO-1 mRNA appears to indicate higher oxidative stress, it is not yet clear whether it is an advantage to stimulate or inhibit its expression [17].

In the present work, the hepatic encephalopathy group showed significant increase in inducible nitric oxide synthase (iNOS) gene expression and nitrite/nitrate levels in the brain compared to the control group. This observation is in agreement with the work of Jiang et al., [3] who reported that ALF resulting from ischemic liver failure led to increased expression of the genes coding for inducible and endothelial isoforms of NOS in brain.
The authors found that increased expression of iNOS and eNOS isoforms was accompanied by increased brain concentrations of nitrites/nitrates confirming that the increases in NOS isoform expression caused by ALF were sufficient to produce an increase in NO production [3]. In addition, previous studies in different models of ALF resulting from toxic liver injury also showed evidence of oxidative/nitrosative stress in brain [18,19]. It was reported that cultured astrocytes exposed to ammonia displayed nuclear factor kappa B (NF-xB) activation and that NF-xB activation led to up-regulation of iNOS protein expression and the subsequent generation of nitric oxide (NO) [20]. Exposure of cortical astrocytes to combinations of ammonia and recombinant IL-1 beta resulted in significant additive increases in expression of both hemoxgenase-1 (HO-1) and inducible nitric oxide synthase (iNOS) consistent with a role of ammonia and IL-1b in the pathogenesis of oxidative/nitrosative stress [21].

In the present study, the hepatic encephalopathy group showed significant increase in percentage of water content in the brain compared to the control group. In agreement with the results of the present work, it was reported that injection of a large dose of galactosamine (2.5g/kg IP) led to brain oedema 24 hours after injection as a part of hepatic encephalopathy [5,12].

Thus, the present study confirmed the presence of oxidative/nitrosative stress in brain as proved by the increase in heme oxygenase-1 gene expression, iNOS gene expression and nitrite/nitrate levels in the brain and brain oedema as shown by the increase in brain water in this model of hepatic encephalopathy induced by injection of galactosamine.

In the current study, minocycline provided partial protection against galactosamine-induced hepatic injury. Similar to the present work, Jiang et al., [3] reported attenuation of increased circulating levels of ammonia in ALF by minocycline treatment and mentioned that this could be the consequence, at least in part, of effects of the drug on gut ammonia production.

In the present work, minocycline provided partial protection against brain oxidative and nitrosative stress in this animal model of hepatic encephalopathy. These results are in accordance with that obtained by Liu et al., [22] who found that minocycline blocked the activation of microglia, attenuated lead-induced secretion of TNF-\(\alpha\), IL-1 beta and expression of iNOS; and protected co-cultured hippocampal neurons. Our results confirmed the work of several previous studies. Cai et al., [23] found that minocycline down regulated oxidative molecules upstream of NF-xB and reduced iNOS levels in the hippocampus of diabetic rats. It was reported that administration of minocycline led to significant attenuation of the increases in expression of markers of oxidative/nitrosative stress (HO-1 and NOS isoforms) with consequent reduction of brain levels of nitrites/nitrates in ALF rats [3].

Minocycline and other tetracycline derivatives are proposed to attenuate apoptosis and inhibit production of reactive oxygen species via an action on mitochondria [24]. However, our results contradict the findings of Park et al., [25] who reported that minocycline had no effect on the expressions of heme oxygenase-1 and neuroglobin in the ischemic cortex in mice. The discrepancy with our results is probably due to the difference in experimental protocol used and possibly animal species difference.

The results of the current work demonstrated also that minocycline provided protection against brain oedema in this animal model of hepatic encephalopathy. These findings are in line with previous reports that minocycline treatment was accompanied by significant decrease in brain water content of ALF rats and that microglial activation and brain accumulation of inflammatory cytokines were implicated in the pathogenesis of brain edema in ALF [6]. Our results seemed to be consistent with the observations of Wasserman and Schlichter [26] who reported that minocycline treatment is effective in limiting brain edema that accompanies intracerebral hemorrhages.

Thus, in the present animal model, treatment with minocycline prevented oxidative/nitrosative stress in brain and brain oedema. These effects may be due to direct effects of minocycline on brain or secondary to minocycline protective effects on liver and improvement of serum ammonia or both.

The results of the last group of our study demonstrated that vitamin E provided partial protection against galactosamine-induced hepatic injury. These results confirmed the work of Túnez et al., [27] who found that vitamin E treatment subcutaneously at 20mg/kg for 5 days reduced serum ALT, AST and ammonia in fulminant hepatic failure induced by thioacetamide and protected brain against oxidative stress. Treatment with vitamin C and E suppressed arsenic-induced AST elevation in rats [28].
The results of this study showed that vitamin E provided partial protection against brain oxidative and nitrosative stress in this animal model of hepatic encephalopathy. The data reported by Kim et al., [29] showed that vitamin E decreased lipid oxidation and heme oxygenase-1 gene expression in the kidney in an animal model of rhabdomyolysis. Calvisi et al., [30] added further support and mentioned that dietary supplementation with vitamin E suppressed heme oxygenase-1 gene expression, abolished iNOS gene expression and decreased NADPH oxidase level in an animal model of liver cancer.

Ayasolla et al., [31] added support and concluded that treatment of glial cells with vitamin E downregulated the expression of iNOS as well as production of NO.

However, our results contradict the findings of El-Azab et al., [32] who showed no significant change in HO-1 plasma level in the group treated with vitamin E in comparison with the diabetic group. The discrepancy with our results is probably due to the difference in experimental protocol used and possibly species and organ differences.

The results of the current work demonstrated also that vitamin E protected against brain oedema in hepatic encephalopathy. These findings are consistent with those reported by Ikeda et al., [33] who demonstrated that the novel vitamin E derivative, 2-(alpha-D-glucopyranosyl) methyl-2,5,7,8-tetramethylchroman-6-ol, significantly attenuated brain oedema following cryogenic brain injury. In addition, Yang et al., [34] found that the vitamin E-treated traumatic brain injury group had edema but less severe than the group not treated with vitamin E on microscopic level although they appeared grossly similar.

The results of current work revealed that vitamin E prevented oxidative/nitrosative stress in brain and improved brain oedema that occurred in hepatic encephalopathy. These effects may be due to its anti-oxidant effect (impact) on brain or secondary to liver protection.

No significant change was found between vitamin E and minocycline in oxidative and nitrosative stress indicators in the brain in this animal model. Our results confirmed the work of Kraus et al., [35] who found that minocycline had neuroprotective properties in mixed cultures of neural cells exposed to oxidative stress with a potency similar to that of vitamin E and suggested that minocycline’s antioxidant properties could be attributed to the presence of a substituted phenol ring in its chemical structure, similar to that of vitamin E.

In the current study, there was positive correlation between serum ammonia (ug/dl) and heme oxygenase-1 gene expression, i-NOS gene expression, nitrite/nitrate and brain water (%) suggesting that serum ammonia may be involved in pathogenesis of oxidative and nitrosative stress in the brain and brain oedema.

There was no significant correlation between brain water and i-NOS gene expression, heme oxygenase-1 gene expression and nitrite/nitrate in brain tissues after controlling of serum ammonia but there was significant correlation before controlling of serum ammonia suggesting that these parameters had no role in brain oedema without role of serum ammonia and that increased ammonia level may be the 1st step in producing brain oedema and the protective effects of minocycline and vitamin E on brain oedema may be mainly due to reduction in serum ammonia.

It can be concluded that galactosamine injection produced hepatic encephalopathy as evidenced by increase in serum ammonia and increase in brain water and increase in oxidative/nitrosative stress markers heme oxygenase-1 gene expression, iNOS gene expression and level of nitrite/nitrate in brain 24 hours after injection. Treatment with minocycline or vitamin E provided partial protective role against hepatic encephalopathy as evidenced by decrease in brain water, oxidative/nitrosative stress in brain. They also, provided partial protection of liver as evidenced by decrease in serum ammonia. Furthermore, no difference between treatment with minocycline or vitamin E in prevention of hepatic encephalopathy except that dexamethasone was the least effective one in reducing serum ammonia.

Finally, ammonia increase may be the 1st step in brain oedema and markers of brain inflammation and oxidative stress may be mediators by which ammonia produces oedema or may be parallel outcomes with oedema in response to increase ammonia.

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