Towards Understanding the Mechanisms of Action of L-carnitine and Alpha Lipoic Acid in Counteracting Hepatic Encephalopathy

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ABSTRACT

Hepatic Encephalopathy (HE) refers to brain function deterioration that occurs as a result of either acute liver failure or chronic liver disease. The main target of the present work was to gain a better understanding of the possible mechanisms by which L-carnitine, α-lipoic acid either individually or in combination can offer their therapeutic action against hepatic encephalopathy in adult male rats. Sixty adult male albino rats were enrolled in the current investigation. They were distributed into six groups: Group (1) control group, Group (2) thioacetamide (TAA) group, Group (3) lactulose-treated group, Group (4) L-carnitine-treated group, Group (5) α-lipoic acid-treated group and Group (6) L-carnitine+α-lipoic acid-treated group. At the end of the experimental period (three months), liver functions test and serum, hepatic as well as brain ammonia levels were quantified. Hepatic and brain oxidant/antioxidant status was evaluated and serum inflammatory markers were quantified. Additionally, histological examination of liver and brain tissue was carried out. The findings of this work showed that the treatment with lactulose, L-carnitine, α-lipoic acid or a combined dose of L-carnitine and α-lipoic acid evoked pronounced improvement in the liver functions accompanied with marked modulation in hepatic and brain oxidant/antioxidant homeostasis in addition to obvious blunting in the inflammatory mediators relative to TAA untreated counterparts. Also, the chosen treatments could restore the structural organization of liver and brain. Conclusively, the outcomes of the present study substantiate the therapeutic ability of L-carnitine, α-lipoic acid or their combination to restore the healing process in the experimental hepatic encephalopathy. The mechanisms behind this effect seem to depend on upregulating hepatic clearance of ammonia, retrieving oxidant/antioxidant homeostasis and downregulating inflammatory status.

Keywords: Hepatic encephalopathy, L-carnitine, α-lipoic acid, Oxidative stress, Inflammation, Rats

INTRODUCTION

Hepatic Encephalopathy (HE) is a neuropsychiatric syndrome that occurs in both cirrhosis and Acute Liver Failure (ALF). Clinically, HE can be diagnosed and a mild-to-moderate grade of the disease may be in a considerable proportion of ambulatory patients with cirrhosis. It is manifested by personality alterations, intellectual impairment, and a suppressed level of consciousness [1]. Fatigue is frequently mentioned in HE and can be correlated with hyperammonemia [2].

Ammonia is defined as a crucial component in the pathogenesis of HE, however many factors, such as oxygen free radicals, circulating levels opioid peptides, nitric oxide, inflammatory mediators, depression in serotonergic neurotransmitters, decline of endogenous antioxidants, neurosteroids and manganese, are also contributed in the development of the disease [3].

Cerebral oedema is also participated in HE, however, the precise pathophysiologic mechanisms responsible for cerebral oedema in HE have not been understood fully. The main hypothesis suggested a state of hyperammonemia which is thought to be the chief causative factor for both direct and indirect alterations in cerebral metabolism [4]. Other mechanisms have been proposed to explain the pathogenesis of HE, including dysfunction of the immune and neurotransmitter systems [5]. Also, inflammation plays a significant role in the molecular pathogenesis of HE and brain oedema [6,7].

At present, there are few therapeutic approaches, and the outcome expectations for patients with HE are poor. Lactulose is a synthetic, non-digestible sugar used in the treatment of chronic constipation and hepatic encephalopathy. It is a disaccharide (double-sugar) formed from simple sugars (monosaccharide) fructose and galactose. It is commercially produced by isomerization of lactose [8]. Lactulose has been found to enhance quality of life and cognitive performance in patients with cirrhosis and minimal hepatic encephalopathy [9].

L-carnitine has been proposed as a therapeutic candidate in metabolic encephalopathies, such as ammonia precipitated hepatic encephalopathy [10].
On the experimental level, L-carnitine has been found to mitigate neurotoxic impact of ammonia, elevate energy metabolism and reduce mortality [11]. A protective influence of L-carnitine against ammonia- precipitated HE has also been demonstrated in cirrhotic patients [12,13]. Alpha-lipoic acid (1,2-dithiolane-3-pentanoic acid; ALA), a disulphide derivative of octanoic acid, and its reduced form dihydrolipoic acid (DHLA), are natural compounds widely distributed in plants and animals. They are synthesized through a reaction catalyzed by lipoic acid synthase within the mitochondria [14]. ALA had been shown to play a protective role against acetaminophen-induced hepatic and renal damage [15]. The action of ALA depends on unique antioxidant ALA/DHLA system. DHLA is capable to inhibit not only reactive oxygen species (ROS) but also oxidize forms of other antioxidants. ALA regenerates other antioxidants and for this reason it is called an antioxidant of antioxidants [16]. Therefore, dietary lipoic acid is effective in ameliorating oxidative stress produced by drugs [17] and aging [18].

The present study was initiated to explore the probable mechanisms underlying the therapeutic ability of L-carnitine and alpha lipoic acid in retrogression of hepatic encephalopathy induced chemically in adult male rats.

MATERIALS AND METHODS

Materials

Chemicals and drugs

Thioacetamide (TAA) was purchased from Sigma Chem. Co., St. Louis, Mo, USA as a pure crystal. It was dissolved in saline and freshly prepared prior to each injection. Lactulose was purchased from Egyptian International Pharmaceutical Industries Co. (EIPICO), Egypt under license of HEK Pharma, Germany. It is manufactured as syrup; each 100 ml contain 65 g of Lactulose. L-carnitine was purchased from MEPCO, Egypt. It is manufactured as capsules; each contains 350 mg of L-carnitine. α-lipoic acid was purchased from EVA PHARMA, Egypt. It is manufactured as capsules; each contains 600 mg of α-lipoic acid.

Experimental animals

Sixty adult male albino rats of Wister strain weighing 120-150 g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimatized in the animal-holding room under controlled environmental conditions (12/12 h light/dark cycle) where temperature 24 ± 1°C and humidity 55% at National Research Centre Animal Facility Breeding Colony. The animals received a standard pellet diet and tap water ad libitum. All animals received human care in compliance with the guidelines of the Ethical Committee of Medical Research of the National Research Centre, Giza, Egypt.

Experimental design

After one week acclimatization, the animals were divided into six groups (10 rats/group). Group 1: The animals in this group received daily 1 ml physiological saline intraperitoneally for 3 days and left untreated for 3 months. This group was assigned as control group. Group 2: The animals in this group were intraperitoneally injected with 100 mg/kg b.wt. of Thioacetamide (TAA) dissolved in physiological saline daily for 3 consecutive days and left untreated for 3 months [19]. This group was assigned as TAA group. Group 3: The animals in this group were intraperitoneally injected with 100 mg/kg b.wt. of TAA dissolved in physiological saline daily for 3 consecutive days and then treated orally with lactulose (8 ml/kg b.wt.) daily for 3 months [20]. This group was assigned as TAA+lactulose group. Group 4: The animals in this group were intraperitoneally injected with 100 mg/kg b.wt. of TAA dissolved in physiological saline daily for 3 consecutive days and then treated orally with L-carnitine (100 mg/kg b.wt.) daily for 3 months [21]. This group was assigned as TAA+L-carnitine group. Group 5: The animals in this group were intraperitoneally injected with 100 mg/kg b.wt. of TAA dissolved in physiological saline daily for 3 consecutive days and then treated orally with α-lipoic acid (100 mg/kg b.wt.) daily for 3 months [22]. This group was assigned as TAA+α-lipoic acid group. Group 6: The animals in this group were intraperitoneally injected with 100 mg/kg b.wt. of TAA dissolved in physiological saline daily for 3 consecutive days and then treated orally with a combined dose of both L-carnitine and α-lipoic acid daily for 3 months. This group was assigned as TAA+L-carnitine+α-lipoic acid group.

Samples collection

At the end of treatment period, the animals were kept fasting for 12 h and blood samples were collected from the retro-orbital venous plexus under diethyl ether anaesthesia. The blood samples were centrifuged at 1800 × g for 15 min at 4°C. Serum was separated and stored at -20°C for biochemical analyses. After blood collection, all animals were sacrificed by decapitation and the whole liver and brain of each animal were rapidly dissected, thoroughly washed with isotonic saline and blotted dry. Liver and brain samples were weighed and sagitally divided into two halves. One half of each liver was immediately homogenized in 50 mM ice-cold phosphate buffer (pH 7.4) to give 20% homogenate (w/v) [23]. While, brain was immediately homogenized to give 10% (w/v) homogenate in an ice-cold medium containing 50 mM Tris-HCl and 300 mM sucrose, pH 7.4 [24]. The homogenates were centrifuged at 1800 × g for 10 min in cooling centrifuge at 4°C. The supernatants of the liver and brain tissue homogenate were separated for biochemical analyses. The other half of each liver and brain was fixed in formalin buffer for histological investigation.

Methods

Biochemical analyses

The activity of serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) was determined colorimetrically using commercial kits obtained from Biodiagnostic Co., Egypt, according to the method of Reitman and Frankel et al. [25]. The level of total bilirubin was assayed colorimetrically using a commercial kit from Biodiagnostic Co., Egypt, following the method of Malloy and Evelyn [26]. Serum total protein was determined by colorimetric method using kit purchased from Biodiagnostic Co., Egypt, according to the method described by Gornal et al. [27]. The level of serum albumin was determined using commercial kit purchased from Biodiagnostic Co., Egypt, following the method of Montgomery et al. [28]. Ammonia concentrations were quantified in serum and homogenates of liver and brain by colorimetric method using kit purchased from Randox, UK, according to the method described by Mondzac et al. [29]. Nitric oxide (NO) levels in liver and brain homogenates were detected colorimetrically using a kit purchased from Biodiagnostic Co., Egypt, following the method described by Satoh [31].
Superoxide Dismutase (SOD) activity in liver and brain homogenates was determined by colorimetric method using superoxide dismutase assay kit purchased from Biodiagnostic Co., Egypt, according to the method of Nishikimi et al. [32]. Glutathione Peroxidase (GPx) activity in liver and brain homogenates was determined by colorimetric method using superoxide dismutase assay kit purchased from Biodiagnostic Co., Egypt, according to the method of Paglia and Valentine [33]. Serum level of tumor necrosis factor-α (TNF–α) was measured by enzyme-linked immunosorbent assay (ELISA) using commercial kits purchased from Koma Biotech Co., Korea according to the manufacturer’s instructions. The level of soluble protein-100-β (S100-β) in serum was assayed by the aid of ELISA using commercial kits (ELAab, USA) according to the manufacturer’s instructions.

### Histopathological investigation

The liver and brain tissues were fixed in buffer formalin for 24 h. The specimens were washed in tap water, dehydrated in ascending grades of ethanol, cleared in xylene and embedded in paraffin wax (melting point 55–60°C). Sections of 4 µ thickness were prepared using sledge microtome and stained with haematoxylin and eosin [34] for examination through the light electric microscope.

### Statistical analysis

In the present study, the results are expressed as Mean±S.E. of the mean. Data were analyzed by one way Analysis of Variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14. The level of significance was set at P value <0.05. Percentage difference representing the percent of variation with respect to TAA group was calculated using the following formula:

\[
\% \text{ difference} = \frac{\text{Treated value} - \text{TAA value}}{\text{Treated value}} \times 100
\]

### RESULTS

#### A Biochemical results

In comparison with TAA group, significant decrease (P<0.05) in serum ALT and AST activity is recorded in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid (data not shown). The percentages of change for ALT are -56.46%, -47.04%, -41.48% and -51.27% and for AST were -63.33%, -51.40%, -42.52% and -55.07% in lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid-treated groups respectively as compared to those in the TAA group (Figure 1). Similarly, significant reduction (P>0.05) is detected in serum bilirubin level in the groups treated with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid versus TAA group (data not shown). The percentages of change are -51.98% for lactulose, -37.85% for L-carnitine, -32.20% for α-lipoic acid and -46.33% for a combined dose of L-carnitine and α-lipoic acid with respect to that in TAA group (Figure 1).

On the other side, treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid leads to significant (P<0.05) elevation in serum levels of TP and ALB in comparison with TAA group (data not shown). The percentages of change for TP are 43.23%, 34.685%, 31.59%, 56.46% and for ALB are 85.19%, 52.91%, 32.28% and 58.73% for lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid-treated groups respectively as compared to those in TAA group (Figure 1).

![Figure 1](image.png)

**Figure 1:** Effect of treatment with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid on serum ALT (U/L), AST (U/L), TP (g/dL), ALB (g/dL) and bilirubin (mg/dL) levels of rats bearing hepatic encephalopathy induced by thioacetamide represented as percentages of change.

Significant decrease (P<0.05) in serum ammonia concentration is observed in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid versus TAA group (data not shown). The percentages of change for serum ammonia are -57.43%, -37.16%, -27.70%, -49.32% in lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid-treated groups respectively as compared to that in TAA group (Figure 2). In the same manner, treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid results in significant decrease (P<0.05) in liver ammonia concentration in comparison with TAA group (data not shown) with percentages of change -57.47% for lactulose, -49.74% for L-carnitine, -45.88% for α-lipoic acid and -52.32% for a combined dose of L-carnitine and α-lipoic acid with respect to that in TAA group (Figure 2).
The same trend is observed upon treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid concerning brain ammonia concentration as these treatments display significant decrease (P<0.05) in brain ammonia concentration in comparison with TAA group (data not shown). The percentages of change are -42.86%, -13.39%, -11.615% and -26.79% in lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid-treated group respectively, as compared to that in TAA group (Figure 2).

![Figure 2: Effect of treatment with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid on serum (μmol/ml), liver and brain (μmol/g wet tissue) ammonia concentrations of rats bearing hepatic encephalopathy induced by thioacetamide represented as percentages of change.](image)

Significant suppression (P<0.05) in liver MDA and NO levels is recorded in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α- lipoic acid relative to TAA group (data not shown). The percentages of change for MDA are -52.13%, -42.57%, -38.725% and -46.34%, and for NO are -35.29%, -27.98%, -25.43% and -31.21% in lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid-treated groups respectively, as compared with those in TAA group (Figure 3). On the contrary, treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid induces significant elevation (P<0.05) in liver GPX activity (data not shown) with percentages of change 50.77% for lactulose, 20 % for L-carnitine, 7.69% for α-lipoic acid and 30.77% for the combined dose of L-carnitine and α-lipoic acid with respect to that in TAA group (Figure 3). By the same way, significant amplification (P<0.05) is detected in liver SOD activity in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid (data not shown) with percentages of change 27.06% for lactulose, 12.62% for L-carnitine, 6.84% for α-lipoic acid and 18.39% for a combined of L-carnitine and α-lipoic acid versus that in TAA group (Figure 3).

![Figure 3: Effect of treatment with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid on liver MDA (nmol/mg protein) and NO (μmol/mg protein) levels as well as GPX (U/mg protein) and SOD (U/mg protein) activities of rats bearing hepatic encephalopathy induced by thioacetamide represented as percentages of change.](image)

Treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α- lipoic acid elicits significant drop (P<0.05) in brain MDA and NO levels compared with TAA group (data not shown). The percentages of change for MDA are -37.98% for lactulose, -30.31% for L-carnitine, -22.56% for α-lipoic acid, and -34.11% for a combined dose of L-carnitine and α-lipoic acid and for N-31.57%, -23.50%, -21.02% and -26.17% for lactulose, L-carnitine and α-lipoic acid and the combined dose of L-carnitine and α- lipoic acid respectively as compared to those in TAA group (Figure 4).

Interestingly, treatment with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α- Lipoic acid produces significant enhancement (P<0.05) in brain GPX activity (data not shown) with percentages of change 94.64% for lactulose, 48.21% for L-carnitine, 33.93% for α-lipoic acid and 60.71% for a combined dose of L-carnitine and α-lipoic acid relative to that in TAA group. On the same line, significant elevation (P<0.05) is detected in brain SOD activity in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid (data not shown) with percentages of change 100.7%, 43.88%, 29.49% and 65.475% for lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid respectively versus that in TAA group (Figure 4).
Figure 4: Effect of treatment with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid on brain MDA (nmol/mg protein) and NO (μmol/mg protein) levels as well as GPX (U/mg protein) and SOD (U/mg protein) activities of rats bearing hepatic encephalopathy induced by thioacetamide represented as percentages of change.

In comparison with TAA group, significant decrease (P<0.05) in serum TNF-α and S100-β levels is recorded in the groups treated with lactulose, L-carnitine, α-lipoic acid or the combined dose of L-carnitine and α-lipoic acid (data not shown). The percentages of change for TNF-α are -55.79%, -44.76%, -40.34% and -49.16%, and for S100-β are -60.19%, -54.34%, -52.88% and -57.23% for lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid respectively when compared to those in TAA group (Figure 5).

Figure 5: Effect of treatment with lactulose, L-carnitine, α-lipoic acid and the combined dose of L-carnitine and α-lipoic acid on serum TNF-α (pg/ml) and S100-β (pg/ml) levels of rats bearing hepatic encephalopathy induced by thioacetamide represented as percentages of change.

Histological results

Liver

Histological examination of liver tissue section of rat in the normal control group shows normal histological structure of the portal area and the surrounding hepatocytes (Figure 6) [35]. Photomicrographs of liver tissue sections of rat in TAA group show fibrosis with inflammatory cells infiltration surrounding the central vein and extended between the hepatocytes (Figures 7a and 7b) [35]. Also, congestion in the portal vein, newly formed bile ductless and periductal fibrosis with oedema in the portal area are observed (Figure 7c) [35].

Photomicrographs of liver tissue sections of rat in the group administered thioacetamide and treated with lactulose show inflammatory cells infiltration in the portal area (Figures 8a and 8b).

Photomicrograph of liver tissue section of rat in the group administered thioacetamide and treated with L-carnitine shows severe congestion in the portal vein and inflammatory cells infiltration in the portal area (Figure 9).

Photomicrograph of liver tissue section of rat in the group administered thioacetamide and treated with α-lipoic acid shows severe congestion in the portal vein with oedema and periductal fibrosis in the portal area (Figure 10). Photomicrograph of liver tissue section of rat in the group administered thioacetamide and treated with L-carnitine+α-lipoic acid shows congestion in the portal vein, and hepatic sinusoids (Figure 11).

Figure 6: Photomicrograph of liver tissue section of rat in the normal control group showing normal histological structure of the central vein (cv) and surrounding hepatocytes (h) (H&E X64) [35]
Figure 7: Photomicrographs of liver tissue sections of rat in TAA group [35] showing: (a) fibrosis (f) with inflammatory cells infiltration (m) surrounding the central vein and extended between the hepatocytes (H&E X64), (b) magnification of (Fig. 7a) to identify the fibrosis (f) and inflammatory cells infiltration (m) surrounding the central vein (H&E X80), (c) Congestion in the portal vein (pv), newly formed bile ductless (b) and periductal fibrosis (f) with oedema (o) in the portal area (H&E X40).

Figure 8: Photomicrographs of liver tissue section of rat in TAA group treated with lactulose showing: (a) Inflammatory cells infiltration in the portal area (m) (H&E X64), (b) Magnification of (Figure 8a) to identify the inflammatory cells infiltration in the portal area (m) (H&E X80).

Figure 9: Photomicrograph of liver tissue section of rat in TAA group treated with L-carnitine showing severe congestion in the portal vein (pv) and inflammatory cells infiltration (m) in the portal area (H&E X80).

Figure 10: Photomicrograph of liver tissue section of rat in TAA group treated with α-lipoic acid showing severe congestion in the portal vein (pv) with oedema (o) and periductal fibrosis (f) in the portal area (H&E X40).
Brain

Microscopic examination of brain tissue sections of rat in the normal control group shows normal histological structure of the meninges and cerebral cortex with highly active nerve cells (neurons) that have huge nuclei with relatively pale stain. The nuclear chromatin and the prominent nucleoli of these cells are apparent. The surrounding supporting cells (glial cells) appear with dense stain, small nuclei and condensed chromatin with no visible nucleoli. The background substances (neuropil) are shown in the cortex. The neurons and vessels exhibit a regular arrangement with distinct edges, and clear nuclei and nucleoli. The glial cells and neuropil are apparent. No necrosis of pyramidal neurons is found (Figure 12a). Normal histological structure of hippocampus (Figure 12b), cerebellum (Figure 12c), and medulla oblongata (Figure 12d) is observed [35].

Microscopic investigation of brain tissue sections of rat in TAA group shows congestion in cerebral blood vessels (Figure 13a), focal and diffuse gliosis in the cerebrum (Figure 13b) and congestion in the capillary plexus (Figure 13c) associated with focal gliosis in the cerebrum (Figure 13d) and focal hemorrhage in the medulla oblongata (Figure 13e) [35]. Microscopic examination of brain tissue section of rat in the group administered thioacetamide and treated with lactulose shows perivascular oedema in the hippocampus (Figure 14).

Microscopic investigation of brain tissue sections of rat in the group administered thioacetamide and treated with L-carnitine shows congestion in blood capillaries and diffuse gliosis in cerebrum (Figure 15a and 15b). Microscopic examination of brain tissue section of rat in the group administered thioacetamide and treated with α-lipoic acid shows focal endothelial swelling and sclerosis in the wall of blood vessels in the medulla oblongata (Figure 16). Microscopic investigation of brain tissue section of rat in the group administered thioacetamide and treated with L-carnitine+ α-lipoic acid shows focal gliosis in the inferior cerebrum (Figure 17).

Figure 11: Photomicrograph of liver tissue section of rat in the TAA group treated with L-carnitine+α-lipoic acid showing congestion in the portal vein (pv) and hepatic sinusoids (s) (H&E X64)

Figure 12: Photomicrographs of brain tissue sections of rat in the normal control group [35] showing:
- Normal histological structure of the meninges (m) and cerebral cortex (cc) (H&E X40),
- Normal histological structure of hippocampus (hp) (H&E X64),
- Normal histological structure of cerebellum (H&E X40),
- Normal histological structure of the medulla oblongata (mo) (H&E X40)
Figure 13: Photomicrograph of brain tissue sections of rat in TAA group [35] showing:
(a) congestion in cerebral blood vessels (v) (H&E X64), (b) focal and diffuse gliosis (g) in the cerebrum (H&E X64), (c) congestion in capillary plexus (cp) (H&E X40), (d) focal gliosis in cerebrum (g) (H&E X64), (e) focal haemorrhage (h) in the medulla oblongata (H&E X64)

Figure 14: Photomicrograph of brain tissue sections of rat in TAA group treated with lactulose showing perivascular oedema (arrow) in the hippocampus (H&E X40)

Figure 15: Photomicrographs of brain tissue sections of rat in TAA group treated with L-carnitine showing:
(a) Congestion in blood capillaries (v) and diffuse gliosis in cerebrum (H&E X40), (b) magnification of (Figure 15a) to identify the congested blood capillaries (v) and diffuse gliosis (arrow) in the cerebrum (H&E X40)
**DISCUSSION**

In the present investigation the focus of our interest was to justify the underlying mechanisms responsible for the favorable impacts of L-carnitine and α-lipoic acid treatment in hepatic encephalopathy. In our previous study, TAA administration in rats caused significant increase in serum ALT and AST activities as well as serum bilirubin level versus controls. Also, TAA administration resulted in significant reduction in the serum levels of total protein and albumin relative to controls. Moreover, significant elevation in serum, hepatic and brain ammonia levels in TAA administered rats with respect to controls has been recorded. Furthermore, oxidant/antioxidant homeostasis has been affected by TAA administration as evidenced by significant elevation in MDA and NO levels of liver and brain tissues of TAA group relative to the control group. On the contrary, liver and brain GPX and SOD activities were dropped significantly in TAA-group versus the control group. Finally, TAA administration in rats experienced significant enhancement in serum levels of TNF-α and S100-β relevant to those in the controls. The explanation of all these observations is well discussed in our previous work [35].

In the current work, great improvement in liver function tests was observed as a result of treatment of TAA group with lactulose compared with TAA group. This finding is in conformity with Jia and Zhang [20]. Lactulose experienced marked improvement in liver functions as indicated by the detectable decrease in serum ALT, AST and bilirubin levels [20]. These data was also supported by the study of Fan et al. [36]. This effect may be attributable to a decline in the absorption of ammonia by conversion of ammonia to the poorly absorbable ammonium ion and to suppression in ammonia production in situ by bacteria [37]. Lactulose elicited significant reduction in serum, liver and brain ammonia levels as shown in the present study. These findings come in line with those of Jia and Zhang [20] who reported that lactulose can significantly lower the level of hyperammonemia and hyper-endotoxemia, lighten Centro lobular necrotic areas as well as inflammatory reaction in rat liver. Lactulose like probiotics in decreasing total ammonia in portal blood by inhibiting bacterial urease activity, suppressing ammonia absorption via dropping pH, diminishing intestinal permeability and improving nutritional status of gut epithelium.

In the present investigation, lactulose could significantly reduce pro-oxidants (MDA and NO) in concomitant with activation of the antioxidant enzymes (GPX and SOD). Lactulose has been shown to reduce oxidative stress in hepatocytes causing an increase in hepatic clearance of ammonia in HE rat model [20].

Lactulose can dramatically induce hydrogen by bacterial decomposition [38] with longer maintenance time of hydrogen concentration [39]. A growing body of evidences has indicated that hydrogen elevates the levels of heme oxygenase-1 (HO-1), catalase (CAT) and SOD [40,41] which are the main downstream antioxidant enzymes in the antioxidant defense system. Lactulose and molecular hydrogen has been found to activate the expression of Nrf2 and thus they could indirectly activate the endogenous antioxidant system. As Nrf2, a positive regulator of the antioxidant response element discovered by Venugopal and Jaiswalin [42], regulates the expression of hundreds of genes, including the familiar antioxidant genes conferring protection against tissue injury [43]. Thus, lactulose oral administration could increase SOD activity, improve the general oxidative stress condition and antioxidant status of the brain [44].
The anti-inflammatory activity of lactulose has been demonstrated in the current study by down-regulation of serum TNF-α and $\text{S100β}$ levels in TAA-challenged group relative to the untreated TAA group. In accordance with our results, Fan et al. [36] reported that lactulose reduces hepatic inflammation scores suggesting that lactulose can ameliorate hepatic inflammation. This finding is greatly supported by the study of Jia and Zhang [20] who stated that lactulose can reduce inflammation in hepatocytes of rats bearing HE.

L-carnitine is an essential intracellular component synthesized from peptide-bound lysine in the liver, brain and kidney [45]. The present data demonstrated that administration of L-carnitine in TAA-challenged group significantly decreased serum AST, ALT activity and bilirubin level. Meanwhile, it significantly increased serum total protein and albumin levels. These findings echo those of Yazar et al. [46], Demiroen et al. [47] and Heibashy et al. [10]. L-carnitine has been found to have a hepatoprotective effect against acute acetaminophen toxicity [46], improves mitochondrial function by enhancing fatty acid oxidation in nonalcoholic fatty liver disease [48] and enhances liver regeneration in rats after hepatectomy [49]. The mechanism underlying the hepatoprotective effect of L-carnitine might be attributed to the direct antioxidant action of L-carnitine. L-carnitine itself was also found to be an antioxidant agent. This property of L-carnitine enables it to protect plasma membranes from the attacking of free radicals and prevent their damage. Consequently, it could reduce the releasing of AST and ALT into the blood stream [50].

In view of the obtained data, oral administration of L-carnitine in TAA-challenged group caused significant reduction in serum, liver and brain ammonia levels. In initial clinical studies, Del Olmo et al. [51] investigated the influence of L-carnitine on hyperammonemia (HA) in patients with cirrhosis, submitted to a rectal ammonium overload test. The results of that study gave the first evidence on the effect of L-carnitine on ammonia clearance. Many studies have demonstrated that L-carnitine can prevent acute ammonia toxicity and reduce its effects on the brain [52-54]. These investigators suggested that this effect of L-carnitine is mainly based on the significant improvement in the cellular redox state and mitochondrial energy metabolism [55]. Moreover, L-carnitine was capable to retrieve the availability of free CoA and thereby could drop ammonia decreases and improve energy metabolism dependent on cerebral cholinergic parameters in hyperammoniemic mouse [56]. The concomitant decrease in blood and brain ammonia concentrations in a consequence of L-carnitine supplementation as shown in the present study was suggested to be due to potentiation of urea cycle by L-carnitine via carbamyl phosphate synthetase 1 [57,58]. The protective effect of L-carnitine against mental function disorder and ammonia-precipitated encephalopathy was also shown in cirrhotic patients with HE, together with lowered circulating ammonia levels suggesting a systemic effect of L-carnitine. These clinical trials are promising as they clarified that L-carnitine can reduce ammonia levels and improve patient performance in HE [59].

The present results showed that administration of L-carnitine in TAA-challenged group elicited significant suppression in MDA and NO production paralleled by significant elevation in GPX and SOD activity. In accordance with our data, Heibashy et al. [10] cited that L-carnitine can reduce lipid peroxidation via decreasing TNF-α-induced production of ROS by macrophages [60]. L-carnitine has been found to scavenge hydrogen peroxide and superoxide anion effectively [61]. These investigators attributed these activities to the powerful antioxidant properties of L-carnitine. Yazar et al. [46] stated that L-carnitine induces significant inhibition of MDA production in the liver. Thus, L-carnitine treatment potentially protected the liver tissue against oxidative insult. The mechanism behind the protective effect of L-carnitine could be also due to the direct antioxidant effect of L-carnitine. Gharem [62] and Hussein et al. [63] stated that administration of L-carnitine in rats fed a high fructose diet results in significant drop in serum NO level due to the significant down regulation of iNOS expression in the liver. So, L-carnitine is an antioxidant that interferes with the chain reaction of lipid peroxidation and stabilizes the cell membrane. The use of L-carnitine has been proven to be beneficial to reduce oxidative stress that has been shown to damage endothelial relaxation and reduce the level of NO [64].

In accordance with the present results, Abdel-Ghaffar et al. [50] stated that L-carnitine treatment improves hepatic GSH level in methotrexate (MTX)-induced oxidative stress in albino rats. The pre-treatment with L-carnitine limited GSH reduction as well as the depletion in the activities of antioxidant enzymes, glutathione-s-transferase (GST), glutathione reductase (GR), GPX, SOD and CAT. This regulation documented the role of L-carnitine in protecting cells against oxidative damage caused by methotrexate in liver tissue. This effect of L-carnitine could be attributed to its antioxidant and free radical scavenging activities [65]. Moreover, El-Khishin et al. [66] reported that L-carnitine promotes good regeneration of the hepatic antioxidant level depleted by Diclofenac (DCLF) in rats [67]. These results were explained by that L-carnitine could significantly improve the liver anti-oxidant capacity by increasing GSH pool and blockage of free radical production that leads to lipid peroxidation [68]. In general, prevention of GSH depletion might be the most efficient way of direct protection against hepatotoxicity by L-carnitine [46].

With respect to hepatic SOD activity in TAA-challenged rats treated with L-carnitine, the significant elevation in the activity of this enzyme is possibly ascribed to the ability of L-carnitine to reverse the down-regulation of hepatic SOD mRNA suggesting the transcriptional control by L-carnitine [69].

Davis et al. [70] found that L-carnitine treatment restores the antioxidant enzymes activity, reduces cell injury and protects cells against cytotoxicity induced by irradiation. Moreover, Yang et al. [71] determined that L-carnitine ingestion up-regulates the expression of genes related to free-radical scavenger enzymes of mice fed with high fat diet. L-carnitine could also replenish the decrease of SOD activity and this may be related to its direct scavenger effect [72].

Ahmed and Mahmoud [73] has investigated the influence of L-carnitine on the levels of lipid peroxidation and nitrite as well as enzyme activities of SOD and CAT in the rat hippocampus during pilocarpine-induced seizures. Generation of ROS is currently viewed as one of the process through which epileptic activity exerted its deleterious effects on brain [74]. These ROS in the absence of an efficient defense mechanism cause peroxidation of membrane polyunsaturated fatty acids [75]. The reduction in nitrite level, due to administration of L-carnitine, is most readily explained as consequence of inhibition of free radical formation, scavenging ROS and depletion of lipid peroxidation products [76]. These results suggested that oxidative stress mediated by pilocarpine exerted its pathologic effects during seizures and also that the neuroprotective and anticonvulsive role of L-carnitine can be mediated by a reduction in lipid peroxidation and nitrite levels of the brain [77]. Possibly, this effect is due to the modulatory effect of L-carnitine on the antioxidant enzymes (SOD and CAT) in the hippocampus of adult rats. Moreover, the augmented CAT activity in those animals suggests that H_2O_2 generated during superoxide dismutation would not be sufficiently removed from the hippocampus by CAT during acute phase of seizures [78].

Thus, the scavenging of oxygen radicals produces a decrease in H_2O_2 levels generated by superoxide dismutation in the hippocampus, causing increase of the activities of SOD and CAT enzymes as neuroprotective defense mechanism stimulated by L-carnitine.

In accordance of the current results, Mescka et al. [79] proved the ability of L-carnitine to promote the activity of GPX in rat cortex. Hence, these investigators provided a clear evidence for the efficacy of L-carnitine as antioxidant protecting candidate against the oxidative stress promoted by neurotoxic agents. Carnitine is able to enhance GSH level due to its energy-promoting property [80].
The anti-inflammatory activity of L-carnitine has been demonstrated in the present investigation by suppressing serum TNF-α and S100-β levels in TAA-challenged group treated with L-carnitine. These findings come in line with those of Heibashy et al. [10] who reported that L-carnitine therapy in rats challenged by lipopolysaccharide (LPS) leads to remarkable reduction in the levels of cytokines (TNF-α, IL-1β and IL-6). These results suggested that carnitine plays a role in managing the level of circulating cytokines. In fact, Delogu et al. [81] observed that carnitine reduces circulating cytokines in patients underwent surgical operation and also inhibits TNF-α secretion by the stimulated human polymorphonuclear cells [82]. These data were also supported by the recent study of Demiroren et al. [47] who demonstrated that L-carnitine, N-acetyl-L-cysteine and genistein can drop the levels of TNF-α and platelet-derived growth factor-binding protein (PDGF-BP), which was elevated in CCL4-intoxicated animals.

Regarding the inhibitory effect of L-carnitine on serum level of S100-β, this could be explained by the neuroprotective action of L-carnitine [73]. This property of L-carnitine enables it to inhibit neuronal damage induced by TAA with consequent reduction in S100-β production. This is because of S100-β levels are highly correlated with the volume of brain damage [83]. Moreover, the antioxidant and free radical scavenging activity of L-carnitine [62] could be responsible for the regression of S100-β serum level in the present study as the oxidative stress and free radicals production enhance S100-β production from astrocytes.

In light of the obtained data, oral administration of α-lipoic acid caused significant decrease in serum ALT, AST activity and bilirubin level. Meanwhile, it significantly increased total protein and albumin levels. These results converge with those of Saraswathy et al. [84] and Somi et al. [85]. The antioxidant ability of α-lipoic acid and its role in GSH recycling has encouraged its use in liver injury [86]. The effects of α-lipoic acid and dihydrolipoic acid (DHLA) were investigated by Foo et al. [87] against TAA-induced liver fibrosis in rats. It was found that co-administration of α-lipoic acid in rats chronically challenged with TAA inhibits the development of liver cirrhosis, as indicated by the reduction in cirrhosis incidence, hepatic fibrosis and AST as well as ALT activities. This reduction in the enzymes activity confirms that α-lipoic acid is responsible for maintenance of normal structural and architectural integrity of hepatocytes by restricting the leakage of these enzymes which can be accounted for membrane-stabilizing property of α-lipoic acid. It was found that α-lipoic acid inhibits ROS generation and exhibits beneficial role in the treatment of chronic liver diseases. It is well known that α-lipoic acid and its reduced form DHLA reduce oxidative stress by scavenging a number of free radicals in both membrane and aqueous domains and preventing membrane lipid peroxidation and protein damage through the redox regeneration of other antioxidants such as vitamin C and E and increasing intracellular GSH [88].

The beneficial effect of oral supplementation of α-lipoic acid on HE have been indicated by the marked correction of serum, liver and brain ammonia levels as shown in the current work. α-lipoic acid has been found to have a nephroprotective activity which is indicated by the decreasing plasma urea level in uremic rats [89]. This means that α-lipoic acid could increase urea excretion in these rats with consequent modulation of urea cycle and increased utilization of ammonia. This explanation could be documented by the recent study of Kleinkauf-Rocha et al. [90] who demonstrated that α-lipoic acid can increase glutamine synthetase activity significantly. This action of α-lipoic acid restores the deficiency of glutamine synthetase flux into the cells with consequent increment in glutamine production and correction of ammonia utilization. It has been suggested that impaired uptake of ammonia due to microcirculatory disturbances plays an important role in the impaired elimination of ammonia in patients with fibrosis [91]. Ultimately, this leads to the reduction of blood, liver ammonia levels due to the treatment with α-lipoic acid as shown in the present study.

The beneficial effect of α-lipoic acid on brain ammonia level might be explained by its action as NMDA receptor antagonistic [92]. It is well known that ammonia-induced astrocyte swelling is mediated by excess NO which is associated with the over activation of NMDA receptor. Thus, the reduction of brain NO level by α-lipoic acid [93], in addition to blocking of NMDA receptor contribute in regression of brain ammonia in TAA-challenged rats as shown in the present work.

In the present investigation, α-lipoic acid could significantly reduce liver and brain MDA and NO levels while, it could elevate GPX and SOD enzymes activity significantly and prevent membrane lipid peroxidation and protein damage through the redox regeneration of the other antioxidants such as vitamin C and vitamin E and increasing intracellular glutathione [94]. Saraswathy et al. [84] demonstrated that the prophylactic administration of α-lipoic acid in Tamoxifen (TAM)-intoxicated rats produces significant increase in all antioxidant enzymes (GPX, SOD, CAT) and hepatic GSH, with significant decrease in the levels of hepatic lipid peroxidation products, serum transaminases and lactate dehydrogenase (LDH) activity. Furthermore, the ability of α-lipoic acid to counteract bromobenzene BB-induced elevation in MDA is consistent with the findings of other investigators who reported that α-lipoic acid can inhibit the process of lipid peroxidation due to its diithiol nature that renders α-lipoic acid reactive against a number of ROS such as hydroxyl radical, superoxide anion, and alkyl radicals [95].

The diithiol nature of α-lipoic acid is also responsible for the direct scavenging of reactive nitrogen species (RNS) such as peroxynitrite, protecting against peroxynitrite-induced inactivation of α-1-antiproteinase and inhibiting nitration of L-tyrosine. Moreover, the indirect effect of α-lipoic acid on inhibiting NO production from activated macrophages in different hepatic injury models has been studied [96]. This was done on the basis of the involvement of ROS in signal transduction pathways of inducing NF-kappa B (NF-κB) activation, a transcription factor required for the expression of iNOS. As long as, oxidative stress upregulates NF-κB, the antioxidants that can down-regulate NF-κB lead to reducing iNOS expression and further decreasing NO production [97]. Thus, α-lipoic acid is able to decrease the synthesis of NO by preventing the upregulation of iNOS [93].

α-lipoic acid has been found to improve the enzymatic antioxidant defense such as SOD, CAT, and GPX activity [98]. A proposed mechanistic explanation for such effect is through increasing ATP production, which may improve overall protein synthesis in cells, thus elevating enzyme synthesis. Non-enzymatic antioxidants such as GSH, vitamin C and E were also increased by α-lipoic acid supplementation, thus supporting the idea that α-lipoic acid acts as an antioxidant for these compounds enabling them to be recycled [98].

The anti-inflammatory activity of α-lipoic acid has been documented in the current investigation via decreasing serum TNF-α and S100-β levels in TAA-challenged group. In accordance of our results, it has been found that lipoic acid can significantly attenuate lipopolysaccharide (LPS)-induced production of TNF-α in rat kuffer cells.

Thereby, lipoic acid modulates the release of a crucial mediator of inflammatory liver diseases [99]. Moreover, Heibashy et al. [10] mentioned that α-lipoic acid prevents the increase of serum cytokine including TNF-α as well as highlighting the anti-inflammatory influence of α-lipoic acid against LPS-induced liver sepsis. These results indicated the ability of α-lipoic acid to prevent the LPS-necrotic damage in rat livers. In fact, α-lipoic acid-induced potentiation of phosphoinositol-3kinase (PI-3-K/Akt) pathway was responsible for the significant improvement of mice survival after LPS challenge in a model of sepsis [100].
The activation of the PI-3-K-Akt pathway, conferring the inhibitory effect on NF-kB, may also explain the observation of reducing LPS-induced, NF-kB-mediated expression of iNOS and TNF-α in rat Kupffer cells, the resident macrophages of the liver, and in murine RAW 264.7 macrophages by α-lipoic acid [101].

In consistent with the current results, Baydas et al. [102] proved the effectiveness of α-lipoic acid administration in decreasing the expression of glial and neuronal markers including S100-β in diabetic rats. These investigators suggested the potential role of α-lipoic acid in preventing neural injury by inhibiting oxidative stress and suppressing reactive gliosis. Herein, we speculate that α-lipoic acid is able to reduce serum level of S100-β due to its powerful antioxidant activity, potent anti-inflammatory property and significant neuroprotective effect.

Judging from our data, the combination therapy of L-carnitine and α-lipoic acid in TAA-challenged group as a model of HE elicited a maximum amelioration in the all studied parameters. These findings are in conformity with Hagen et al. [103], Alam et al. [68] and Gjumrakch et al. [104]. Indeed, the present results could be attributed to the synergistic effects of both L-carnitine and α-lipoic acid. It has been demonstrated that the combined supplementation of acetyl-L-carnitine at 1.5% wt/vol in drinking water and 0.5% wt/wt α-lipoic acid in diet on 3-5 months old rats results in increased metabolism and decreased oxidative stress measured through mitochondrial membrane potential, O₂ consumption, and lipid peroxidation. These findings were stronger in the combination treatment when compared to either supplement alone, lending evidence to a synergistic action between acetyl-L-carnitine and α-lipoic acid. Interestingly, α-lipoic acid administration in HE rat model leads to improvement of the physiological, biochemical and pharmacological properties of L-carnitine acting together as cell membrane stabilizers antioxidants, free radical scavengers and anti-inflammatory agents to alleviate the damaging insults on liver tissue. Moreover, L-carnitine along with α-lipoic acid was observed to reduce age associated mitochondrial ultra-structural decay leading to improvement of brain function [105].

In our previous work, histological examination of liver tissue section of TAA-challenged rat revealed severe congestion in the portal vein with infiltrate of inflammatory cells. Also, fibroblastic cells proliferation was extended in between the hepatocytes dividing the parenchyma into lobules [35].

Also, in our previous study histological investigation of brain tissue sections of rats in TAA-challenged group revealed focal gliosis in the cerebrum associated with focal haemorrhage in the medulla oblongata [35].

In this study, histological examination of liver tissue section of rat in TAA-challenged group treated with lactulose revealed inflammatory cells infiltration in the portal area. This finding is in respect with William et al. [106] who observed that hepatic parenchyma is replaced by mild mononuclear inflammation and attempted regeneration in the form of zone ductular metaplasia after lactulose supplementation in hepatic fibrosis subjects.

Histological investigation of brain tissue section of rats in TAA-challenged group treated with lactulose showed perivascular oedema in hippocampus. This finding is in agreement with Zhai et al. [107] who reported that the treatment of cerebral ischemia/reperfusion rat model with lactulose markedly increases hydrogen production and improves antioxidant status and histopathological feature of brain tissue. It has been demonstrated that lactulose had potential amelioration of cerebral infraction injury by inducing intestinal hydrogen [108].

Histopathological investigation of liver tissue sections of rats in TAA-challenged group treated with L-carnitine showed sever congestion in portal vein and inflammatory cells infiltration in portal area. These results go hand in hand with the study of Demirdag et al. [109] who reported, that L-carnitine reduces lipid peroxidation, steatosis, inflammation and necrosis in acute liver damage rat model induced by CCLA. Also, Yapar et al. [46] proved the beneficial effect of L-carnitine against acetaminophen hepatotoxicity in mice through the presence of scattered cells in the centrilobular region, cytoplasmic vacuoles in association with the increased eosinophilia in the hepatocytes. Moreover, these investigators observed that the nuclei of these hepatocytes are picnotic with very limited necrosis and hemorrhages. Histological examination of brain tissue sections of rats in TAA-challenged group treated with L-carnitine revealed congestion in blood capillaries and diffuses gliosis in cerebrum. These observations are concordant line with those of El-Maddawy [110] who observed mild edema in purkinje cell layer of the cerebellum and appearance of congestion in some meningeal blood vessels in gentamicin-induced brain damage in rats treated with L-carnitine. Additionally, Silva-Adaya et al. [111] demonstrated that L-carnitine induces significant reduction in the rates of neuronal damage produced by 3-nitropropionic acid in rat brain.

Histological examination of liver tissue sections of rats in TAA-challenged group treated with α-lipoic acid showed sever congestion in the portal vein with oedema and periductal fibrosis in portal area. These findings match those in the study performed by Saraswathy et al. [84] who reported that α-lipoic acid displays some histopathological changes in rat liver represented in hepatic necrosis and congestion in the liver of rats in phenytoin-induced haematoxotoxicity and oxidative stress. Histological examination of brain tissue sections of rats in TAA-challenged group treated with α-lipoic acid showed endothelial swelling and sclerosis in the wall of blood vessels in medulla oblongata. These results fit similar findings reported by Saraswathy et al. [84] who demonstrated that α-lipoic acid exhibits gliosis and congestion in the brain of rats in phenytoin-induced oxidative stress in the brain.

Histological examination of liver tissue sections of rats in TAA-challenged group treated with a combined dose of L-carnitine and α-lipoic acid showed congestion in the portal vein and hepatic sinusoids. These results are comparable to the previous study of Alam et al. [69] who reported that post treatment with L-carnitine and α-lipoic acid in rats exposed to γ-irradiation-induced cellular damage reveals focal hepatic hemorrhage and binucleated hepatocytes in addition to vacuolar degeneration of some hepatocytes. Histological investigation of brain tissue sections of rats in TAA-challenged group treated with combined dose of L-carnitine and α-lipoic acid showed focal gliosis in inferior cerebrum. These findings match those in the study of Gjumrakch et al. [101] who reported that treatment with acetyl L-carnitine+α-lipoic acid ameliorates the age-associated neuronal damage.

CONCLUSION

In conclusion, the results of the present work speak for the importance of ammonia, oxidation stress and inflammation as key players in development of hepatic encephalopathy. Within this context, the present findings justify the therapeutic ability of L-carnitine and α-lipoic acid via mitigation of these key events which may be the probable mechanisms by which these candidates can offer their therapeutic action against hepatic encephalopathy. The outcomes of the present investigation may be clinically beneficial to proceed for further clinical trials.
REFERENCES


