ORIGINAL RESEARCH

The Potential Neuroprotective Role of Citicoline in Hepatic Encephalopathy

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Purpose: Hepatic encephalopathy (HE) is described as impaired brain function induced by liver failure. Ammonia is the most suspected chemical involved in brain injury during HE. Although the precise mechanism of HE is not clear, several studies mentioned the role of oxidative stress in ammonia neurotoxicity. In animal models, the use of some compounds with antioxidant properties was reported to reduce the neurotoxic effects of ammonia, improve energy metabolism, and ameliorate the HE symptoms. Citicoline is a principal intermediate in the biosynthesis pathway of phosphatidylcholine that acts as neurovascular protection and repair effects. Various studies mentioned the neuroprotective and antioxidative effects of citicoline in the central nervous system. This study aims to investigate the potential protective effects of citicoline therapeutic in an animal model of HE.

Materials and Methods: Mice received acetaminophen (APAP,1g/kg, i. p.) and then treated with citicoline (500 mg/kg, i.p) one and two hours after APAP. Animals were monitored for locomotor activity and blood and brain ammonia levels. Moreover, markers of oxidative stress were assessed in the brain tissue.

Results: The result of the study revealed that plasma and brain ammonia and the liver injury markers increased, and locomotor activity impaired in the APAP-treated animals. Besides, an increase in markers of oxidative stress was evident in the brain of the APAP-treated mice. It was found that citicoline supplementation enhanced the animal's locomotor activity and improved brain tissue markers of oxidative stress.

Conclusion: These data propose citicoline as a potential protective agent in HE. The effects of citicoline on oxidative stress markers could play a fundamental role in its neuroprotective properties during HE.

Keywords: antioxidants, citicoline, hepatic encephalopathy, hyperammonemia, oxidative stress

Introduction

Hepatic encephalopathy (HE) described as impaired brain function caused by liver failure manifesting as wide-ranging neurological and psychiatric abnormalities.[1–3] HE occurs in around 40% of patients with cirrhosis. HE has also been reported in about 10–50% of patients who undergo the transjugular intrahepatic portosystemic shunt (TIPS) placement.[4] Although the pathophysiology of HE is complex, it has been found that ammonia could play a pivotal role in this complication.[3,5] On the other hand, oxidative stress is mentioned as a crucial mechanism involved in the pathogenesis of ammonia neurotoxicity.[5] Therefore, various studies tested the potential neuroprotective role of antioxidants in the management of HE.[6,7] In animal models, the use of some compounds with antioxidant properties has been indicated

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to decrease the neurotoxic effects of ammonia. L-carnitine, taurine, carnosine, N-acetyl cysteine, and α-lipoic acid supplementation have been tested as hepatic/neuroprotective agents in animal models or clinical studies.[6–14]

Citicoline is a principal intermediate in the biosynthesis pathway of phosphatidylcholine.[15] Several investigations revealed that citicoline acts as a neurovascular protecting agent.[16] Citicoline is contemplated as an exogenous source of choline and cytidine that could increase the synthesis of acetylcholine in the brain.[17] Citicoline has neuroprotective properties due to some mechanisms like the maintenance of sphingomyelin synthesis and integrity in biomembranes and enhancing the level of cellular antioxidant defense systems.[18]

Previous studies revealed the positive effects of citicoline in the management of several neurological disorders, such as intellectual deficits after brain injury, depression, stroke, and other cardiovascular diseases.[19,20] The effects of citicoline on oxidative stress-associated parameters seem to play a pivotal role in its neuroprotective effects against these diseases.[19,20] On the other hand, there are no studies about the effects of citicoline on patients with HE. Acetaminophen (APAP) is a hepatotoxic agent frequently used as an animal model due to its efficacy in inducing hepatic failure.[21] APAP-induced hepatic failure could lead to hyperammonemia and HE in mice.[21] The current study was designed to investigate the potential protective effects of citicoline against APAP-induced HE.

Materials and Methods
Chemicals
4,2-Hydroxyethyl,1-piperazine ethane sulfonic acid (HEPES), thiobarbituric acid (TBA), 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox), sucrose, fatty acid-free bovine serum albumin (BSA) fraction V, Coomassie brilliant blue, dithiobis-2-nitrobenzoic acid (DTNB), malondialdehyde (MDA), glutathione (GSH), $2',7'$-Dichlorofluorescein diacetate (DCFH-DA), potassium chloride (KCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium chloride, Trichloroacetic acid (TCA), and hydroxymethyl aminomethane hydrochloride (Tris-HCl) were purchased from Merck (Darmstadt, Germany). Citicoline was purchased from Alborz Darou® (Tehran, Iran). Commercial kits were

used for the assessment of plasma biomarkers (Pars Azmoon®, Tehran, Iran).

Animals
Twenty-four male C57BL/6J mice (n = 24) weighing 21 ± 25 g were obtained from the Shiraz University of Medical Sciences, Shiraz, Iran. Animals were kept in a standard environment (The ambient temperature at 23 ± 1 °C, 12L: 12D photo schedule, and 40% of relative humidity). Mice were fed with standard mice chow diet (RoyanFeed®, Isfahan, Iran) and water ad libitum. All processes containing animal use were performed under the ethical guidelines approved by the Ethics Committee of Shiraz University of Medical Sciences (No:19776).

Experimental Design
Acetaminophen (APAP)-induced acute liver failure was accomplished by a single injection (1g/kg, i.p). Citicoline was ordered intraperitoneally one hour after acetaminophen in group III, and one and two hours after acetaminophen in group IV. The treatment groups were as follows: Group I: Control, vehicle, Group II: APAP, 1g/kg, i.p. APAP single dose, Group III: Citicoline-treated group 1: receiving 1g/kg, i.p. APAP + 500 mg/kg, i.p citicoline one hour after APAP, Group IV: Citicoline-treated group 2: receiving 1 g/kg, i.p. APAP + 500 mg/kg, i.p, citicoline one and two hours after APAP.

Animals' locomotor activity was evaluated 24 hours after APAP treatment. Then, animals were anesthetized (thiopental, 80 mg/kg, i.p), and samples were collected.

Animal Behavioral Tests
Rotarod Test
Based on the previous protocols, each animal experienced four sessions of rotarod performance.[22,23] The speed of the rotarod was 10 rpm with a cut-off point 300 sec. Each test period for each animal consisted of three episodes with 10 min intervals. The time that each mice stayed on the rotating rod was automatically recorded.[22,23]

Open-Field Behavior Test
Open-field behavior test was used as an index of animals' locomotor activity in the animal model of hyperammonemia and hepatic encephalopathy.[24] Briefly, the open field apparatus was made of a white Plexiglas box (100 cm L × 100 cm W × 30 cm H), and the box floor was divided into 25 squares (25 ×25 cm). The open-field was equipped with a webcam (2.0 Megapixel, Gigaware, UK), and animals' activities were

monitored and recorded for 15 minutes. The total number of crossed squares (whole locomotion) were counted in each group.[24,25]

Gait Stride Test

Gait stride was assessed as an index of locomotor function in HE.[23,24] For this purpose, mice hind paws were wetted with ink and animals were allowed to walk down on a paper strip (60 cm long, 10 cm wide) from the brightly lit corridor toward the dark side, and the distance between the points of the left and right hind paws was measured.[23,26]

Sampling Methods

Animals were anesthetized (thiopental 80 mg/kg, i.p), and blood samples were collected from the portal vein, transferred to heparin-coated tubes, and centrifuged (1800 g, 15 min, 4°C). The plasma was used for biochemical analyses. The brain and liver were isolated and transferred to a 0.9% N/S container. The pathological finding was detected by H&E staining. Moreover, two pieces of the brain were collected for measurement of ROS formation, GSH content, lipid peroxidation, total antioxidant capacity, and histopathological analysis.

Blood Biochemistry and Ammonia Level

Standard commercial kits and a MindrayBS-200® autoanalyzer were used to measure plasma aspartate aminotransferase (AST), alanine aminotransferase (ALT), and bilirubin. The plasma ammonia level was measured with standard kits based on the method of phenate-hypochlorite reaction.[24] For the determination of brain ammonia content, the sample (100 mg) of the forebrain (cerebral cortex) was collected, homogenized, and deproteinized in 3mL of ice-cooled lysis solution (Trichloroacetic acid, 6%, w/v, 4°C). After centrifugation (17,000g, 10 minutes, 4°C), the supernatant was collected and neutralized with potassium carbonate (100 μL of $KHCO_3$; 2 mol/L, pH = 7). Afterward, brain ammonia content was assessed by standard kits.[24]

Lipid Peroxidation in the Brain Tissue

Thiobarbituric acid reactive substance (TBARS) were measured to estimate lipid peroxidation in the brain tissue. Briefly, tissue samples were homogenized in 5 mL of KCl (1.15%, w: v solution), and then, a homogenous mixture containing 10% tissue was prepared. Afterward, 0.5 mL of brain homogenate was treated with thiobarbituric acid (1 mL of 0.6%, w: v), and 3 mL of meta-phosphoric acid (1% w: v,

pH = 2).[27,28] Samples were mixed well and heated in a water bath (100 °C; 45 min). Then, the mixture was cooled, and 4 mL of n- butanol was added. Samples were vigorously vortexed and centrifuged (10,000 g, 5 min). Finally, the absorbance of developed color in the upper phase (n-butanol phase) was read at λ = 532 nm (Ultrospec 2000®UV spectrophotometer, Pharmacia Biotech, Uppsala, Sweden).[14]

Reactive Oxygen Species (ROS) Formation in the Brain Tissue

2′, 7′-dichlorofluorescein-diacetate (DCF-DA) was used as a fluorescent probe to estimate brain ROS level.[29–31] Brain tissue was weighed in different groups, with a ratio of 1:10 and was homogenized in 5 mL of ice-cooled (4°C) Tris-HCl buffer (40 mM, pH = 7.4). Samples (100 μL) of the resulted tissue homogenate were mixed with Tris-HCl buffer (1 mL; pH = 7.4 4°C) and 5 μL of DCF-DA (Final concentration of 10 μM).[32–34] The mixture was incubated at 37°C (15 min, in the dark). Finally, the fluorescence intensity of the samples was assessed using a fluorimeter (FLUOstar Omega® multi-functional microplate reader, $λ_{excit}$ = 485 nm, and $λ_{emiss}$ = 525 nm).[29,30]

Brain Tissue Glutathione (GSH) Content

Brain tissue glutathione level was assessed using the Ellman's reagent (dithiobis-2-nitrobenzoic acid, DTNB).[35] Briefly, 5 mL of the prepared brain homogenate was treated with 1 mL of trichloroacetic acid (50% w: v; 4°C). Samples were mixed well and centrifuged (3000 g, 4°C, 15 minutes). Afterward, 2 mL of the supernatant was mixed with 4 mL of Tris buffer (pH = 8.9; 4°C), and 0.1 mL of DTNB (dissolved in methanol). Finally, the absorbance of the developed color was measured at λ = 412 nm UV spectrophotometer.[36]

Brain Tissue Antioxidant Capacity

The total antioxidant capacity of the brain tissue was assessed using the ferric reducing antioxidant power (FRAP) test.[37–39] Briefly, 100 μL of the tissue homogenate was added to 1 mL of FRAP reagent (composed of 2.5 mL of acetate buffer 3000 mM, pH: 3, 0.25 mL iron chloride solution, and 0.25 mL of TPTZ solution 10 mM in 40 mM HCl). The mixture was incubated at 37°C (5 min, in the dark). Finally, samples were centrifuged (17,000 for 1 minute), and the absorbance of the developed color was measured at λ = 593 nm UV spectrophotometer.[37,38]

Table 1 Evaluation of Blood Biochemistry Markers and Histopathological Liver Injury Markers in Study Groups

Treatments	Blood Biochemistry			Liver Histopathological Alterations			
	ALT (U/l)	AST (U/l)	Total Bilirubin (mg/dl)	Focal Necrosis	Portal Inflammation	Fatty Changes	Ballooning Degeneration
Control	47±3	73±3	0.09±0.01	-	-	-	-
APAP-treated group	222±31[a]	482±67[a]	0.43±0.03[a]	+ + +	+ + +	+	+
Citicoline-treated group A	134±28[b]	207±64[b]	0.18±0.02[b]	-	+	+	+
Citicoline-treated group B	78±11[b]	147±23[b]	0.16±0.04[b]	+	+	_	+

Notes: Data are presented as mean ± SD (n = 6). [a] Indicates significant difference as compared with the control group (p < 0.05). [b] Indicates significant difference with the APAP-treated group (p < 0.05). [-], [+], and [+++] indicate no significant, mild, and severe histopathological changes, respectively.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; APAP, acetyl-para-aminophenol; acetaminophen.

Statistical Methods

Data are represented as mean±SD. The comparison of data sets was performed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as the post hoc test. Values of P < 0.05 were considered statistically significant. Animal sample size (n = 6) was estimated based on the Cochran method.

Results

Serum Biochemistry

A significant increase in serum level of liver injury biomarkers included serum AST, ALT, and bilirubin were detected in APAP-treated animals (P < 0.05) (Table 1). Meanwhile, levels of these plasma biomarkers of liver injury significantly lower in citicoline-treated groups (Table 1). On the other hand, a high-level of brain tissue and plasma ammonia was detected in the APAP group in comparison with the control group (Table 1), and there were no significant differences in both plasma and brain ammonia levels between citicoline-treated groups and APAP group (Figure 1).

Liver Histopathological Alterations

Based on the histopathological examination, we observed significant hepatic injury in APAP- treated animals (Figure 2 and Table 1). The histopathological alterations involved hepatic necrosis, hydropic changes, portal inflammation, fatty changes, and ballooning degeneration compared with the control group. On the other hand, a lower level of liver tissue histopathological changes was detected in the citicoline-treated mice (Figure 2 and Table 1).
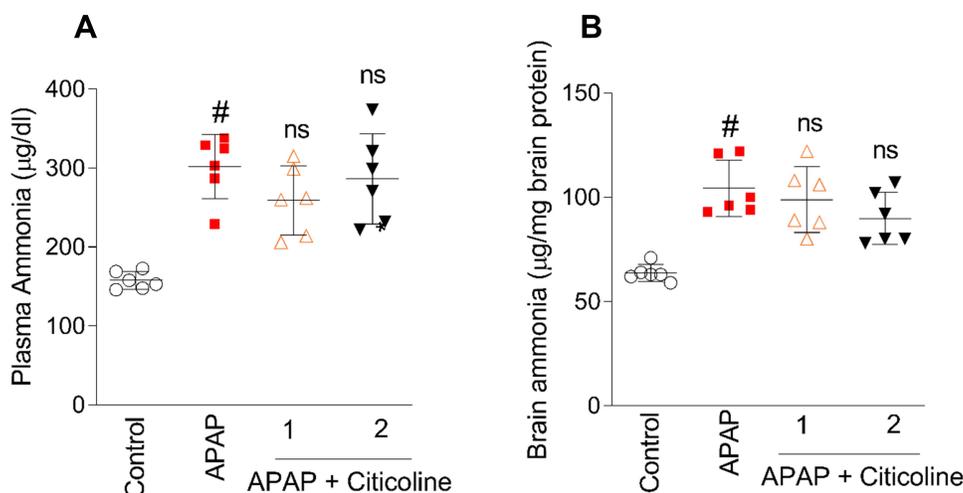

Figure 1 Evaluation of plasma (**A**) and brain tissue (**B**) ammonia levels in mice receiving acetaminophen (APAP) and then citicoline. Citicoline-treated group A (1), Citicoline-treated group B (2).
Notes: Data are presented as mean ± SD (n = 6). **#** Indicates significant difference with control group (P < 0.05).
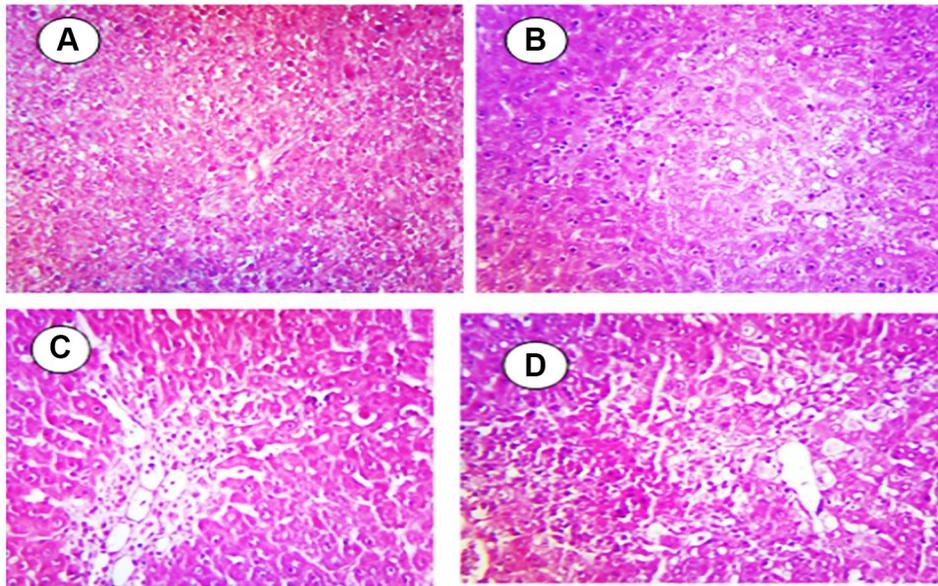**Abbreviation**: ns, not significant, indicates no significant difference with the APAP group.

Figure 2 Histopathological liver examination of different doses of citicoline on acetaminophen-induced liver injury in mice. (**A**) Control group: No changes were observed in this group, (**B**) APAP-toxin group: Liver tissue changes including tissue necrosis, inflammation of the port ducts and fat alterations were found in this group, (**C**) Citicoline-treated group one: The improvement effects of inflammation were observed that it is comparable with those in the APAP-toxin group, (**D**) Citicoline-treated group two: The significant improvement effects of inflammation were observed that it is comparable with those in the APAP-toxin group.

Locomotor Activity in APAP-Treated Mice

We observed that animals' locomotor activity significantly decreased in the APAP -treated groups (P < 0.001) (Figure 3). Lower open-field behavior activity and impaired rotarod and gait stride test were evident in the APAP-treated group (P < 0.001) (Figure 3). It found that citicoline supplementation (500 mg/kg, i.p) enhanced animals' locomotor activity in APAP-treated mice (P < 0.001) (Figure 3).

Brain Markers of Oxidative Stress

Brain tissue markers of oxidative stress and lipid peroxidation were significantly higher in the APAP group as compared with the control group (Figure 4). A lower level of brain tissue GSH content and antioxidant capacity observed in the APAP group in comparison with the control group. In contrast, brain tissue antioxidant capacity was a significant difference and increased in citicoline-treated groups compared with the APAP group. (P < 0.001) (Figure 4).

It found that citicoline administration (500 mg/kg, i.p) significantly alleviated brain tissue biomarkers of oxidative stress (lipid peroxidation, ROS formation, and total antioxidant capacity) in hyperammonemia animals. Besides, a lower level of ROS and lipid peroxidation were identified in citicoline-supplemented mice (Figure 4). Moreover,

brain antioxidant capacity was reserved at a higher level in citicoline-treated animals (Figure 4).

Discussion

HE is a severe clinical complication which needs urgent treatment to prevent permanent brain injury or patients' death.[40,41] Hence, finding new therapeutic approaches to protect the brain tissue in He is of great clinical value. The result of this study showed that a high dose of APAP in mice (1 g/kg, i.p) could be used as a model for studying HE. Liver failure, the significant increase in serum and cerebral ammonia levels, the induction of behavioral changes, and oxidative stress in brain tissue was detected in APAP-treated animals (Figure 5). We observed that citicoline supplementation (one or two doses of 500 mg/kg, i.p) improved locomotor activity and decreased oxidative stress biomarkers in the brain of HE animals. This supplement may be used as a new strategy in the management of HE (Figure 5).

HE is a major CNS complication associated with liver diseases that are described by a wide range of symptoms, such as neurological and neuropsychological impairments.[40,41] Based on the International Society for Hepatic Encephalopathy and Nitrogen Metabolism (ISHEN) criteria, HE is classified into three types.[42] Type A of HE is associated with acute liver failure
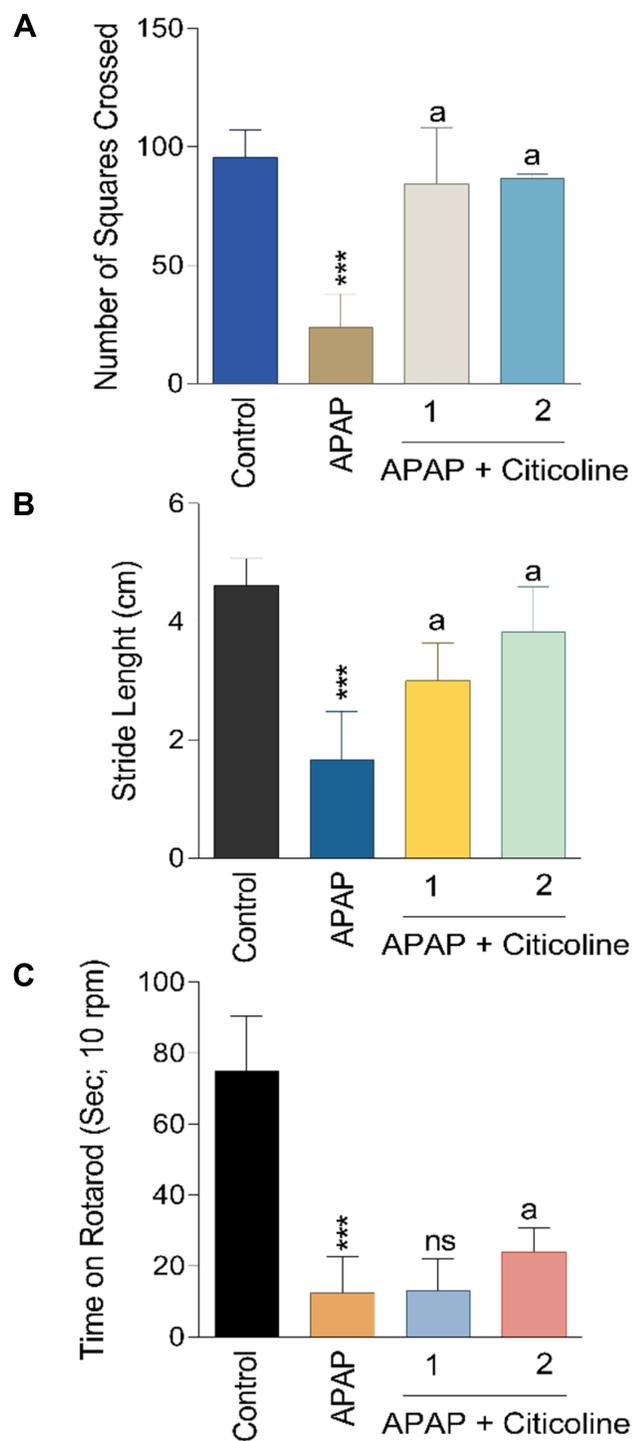
Figure 3 Evaluation of locomotor activity and level of consciousness based on the number of squares crossed (**A**), stride length (**B**), and time on the rotarod (**C**) in mice receiving acetaminophen (APAP) and citicoline. Citicoline-treated group A (1), Citicoline-treated group B (2).
Notes: Data are presented as Mean ± SD (n = 6). *** Indicates significant difference with control group ($P < 0.001$). ^{a} Indicates significant difference with the APAP-treated group ($P < 0.05$).
Abbreviation: ns, not significant, indicates no significant difference with the APAP-treated group.

resulting from severe inflammatory and/or necrotic liver disease of rapid onset. Type B of HE results from porto-caval shunting in the absence of parenchymal liver disease. Type C of HE that accompanies chronic liver failure (cirrhosis). Etiology of cirrhosis resulting from alcohol, viral infection, biliary obstruction, drugs, or toxins. Portal hypertension arises as a result of cirrhosis; the high portal pressure stimulates the opening of embryonic venous channels. Portal-systemic shunts then permit toxins of intestinal origin (examples include ammonia, manganese, cytokines) to bypass the liver into the systemic circulation.[42] As we used a high dose of APAP (1 g/kg), the current model is a type A of HE. High doses of APAP (1 g/kg) is also used to induce acute liver failure in previous investigations.[43]

The primary neurological impairments in HE include the destruction of psychomotor, neurocognitive deficits, and impaired motor function.[40,41] In the current study, we found that brain ammonia levels and some locomotor activity tests, including open-field behavior, rotarod test, and gait stride, were impaired in APAP-treated animals (Figures 1 and 3). These data could indicate a neurological deficit in APAP-treated animals. Although the specific mechanisms of brain injury and other HE still require studies widely, researchers have emphasized the essential role of ammonia in encephalopathy-induced injury.[3,44,45] The liver produces ammonia by the metabolism of amino acids, and it enters the urea cycle, converts to urea, and eventually excreted in the urine. Hepatic dysfunction causes urea cycling dysfunction and elevated plasma ammonia levels, and then, high plasma levels of ammonia can eventually cause coma and death.[46]

Increased levels of ammonia could cause severe oxidative stress, tissue inflammation, edema, and increased intracranial pressure.[3] Exposure to high concentrations of ammonia has been reported to inhibit enzymes involved in the Krebs cycle, inhibition of mitochondrial respiratory chain complexes, induction of oxidative stress, and ultimately disruption of energy metabolism and cell death.[3,47,48] Cellular mitochondrial are also the primary sources of ROS.[49] Therefore, finding the mechanisms of brain injury during HE is a critical step for the development of therapeutic strategies in this disease.

In the present study, it was found that using the acetaminophen model for induction of acute liver failure and
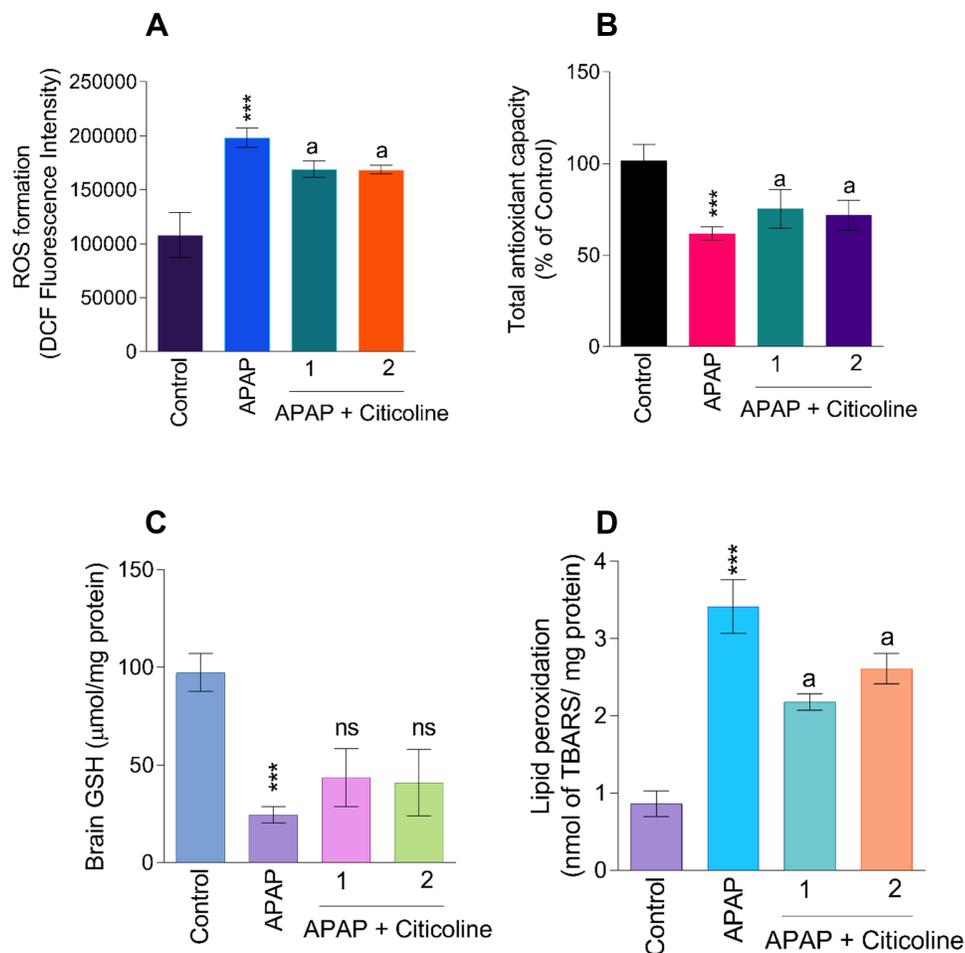
Figure 4 Evaluation of brain tissue ROS formation (**A**), total antioxidant capacity (**B**), brain GSH (**C**), and lipid peroxidation (**D**) in mice receiving acetaminophen (APAP) and citicoline (1= 1 hour after APAP injection, 2 = 1 and 2 hours after APAP injection).

hyperammonemia could lead to significant impairment of glutathione stores, oxidative stress indices, and locomotive activity alterations. These complications are also mentioned in recent studies.[14,21,24] Therefore, the model used in this study was a suitable model of the disease that able to induce liver damage as well as symptoms of encephalopathy and increase ammonia in the brain and damage this organ (Figure 5). Following previous reports which indicate APAP intoxication caused a significant decrease in brain GSH levels as compared to control animals. They had confirmed the occurrence of oxidative stress through a considerable increase of TBARs levels and a substantial decrease of GSH in the liver of APAP-treated animals.[21] Previous studies also confirmed the presence of oxidative stress in the cerebral cortex of hyperammonemia and HE animals.[24,50] It was found that significant oxidative stress occurred in the cerebral cortex, cerebellum, and pons medulla in animals treated with APAP.[21,24,50] Besides, it has been recognized that ROS was more distinct in the

pons, medulla, and cerebral cortex than that present in the cerebellum as they found a substantial increase in MDA and nitric oxide levels with a significant decrease in glutathione ratio and modified antioxidant defense system. In other previous reports, they studied the different oxidant and antioxidant parameters on three different parts of brain tissue in thioacetamide-induced HE in a rat model.[51]

Astrocytes are the main target of ammonia neurotoxicity.[52–54] The precise mechanism of ammonia-induced cellular injury is poorly understood. Studies over the past decade have invoked the concept of oxidative stress as a pathogenic mechanism for ammonia neurotoxicity.[55] In support of the role of oxidative stress in HE is studies showing that treatment of experimental animals with HE/hyperammonemia with antioxidants (eg, ascorbate, α- tocopherol, deferoxamine, butylated-hydroxy anisole, dimethyl sulfoxide, and dimethyl thiourea) had beneficial effects.[55] Antioxidants also have been shown to have valuable effects in experimental models of HE,
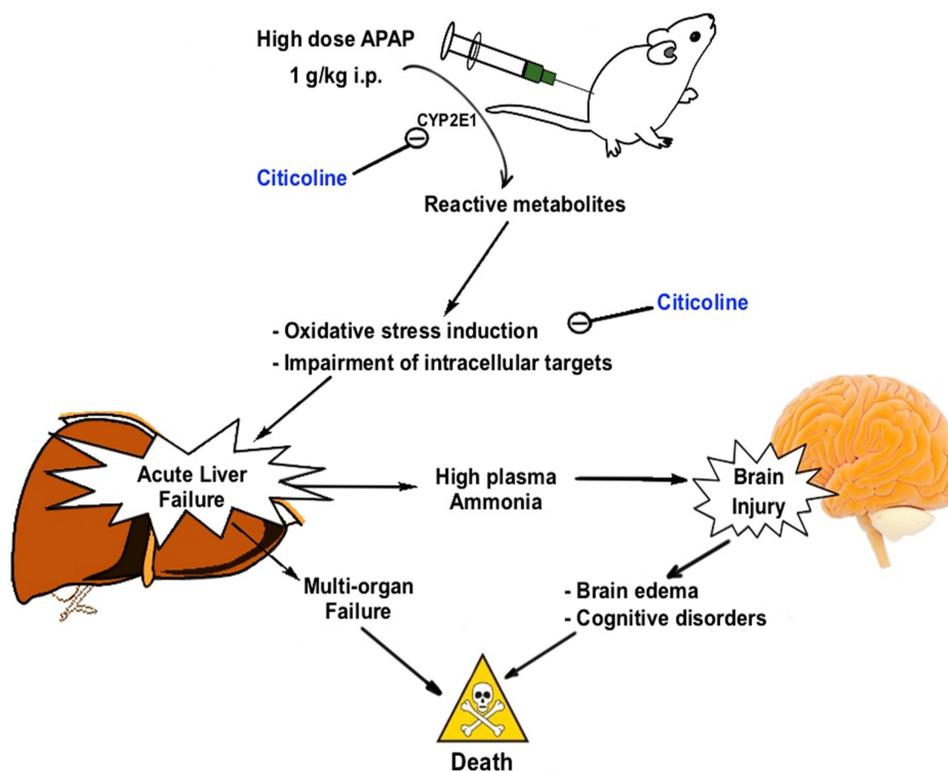
Figure 5 Schematic representation of the effect of citicoline on hyperammonemia and hepatic encephalopathy-induced brain injury.
Notes: Data are presented as mean ± SD (n = 6). *** Indicates significantly different as compared with the control group (P < 0.001). [a]Indicates significantly different as compared with the APAP-treated group (P < 0.05).
Abbreviation: ns, not significant, indicates no significant difference with the APAP-treated group.

as well as in patients with mild or moderate HE. The improvement in mitochondrial function and ameliorating the oxidative stress in the brain tissue is involved in the mechanism of neuroprotection in HE models.[56]

Citicoline is a generic name for a drug that is similar to the natural intracellular phospholipid phosphatidylcholine[57] and appears to stimulate cell membrane stabilization and possibly reduce inflammation.[58] Citicoline has been used as a single treatment or in combination with other medications in several complications such as stroke, cerebrovascular disease, and Parkinson's disease.[16,59,60] Some studies mentioned that only the systemic administration (not topical; eg, eye drops that used for protecting retina) act as a neuroprotective agent.[60,61] Besides, citicoline helps in the synthesis of nucleic acid-protein and acetylcholine and other neurotransmitters and reduces the production of free radicals.[62] In the current study, we found a positive association between citicoline supplementation (500 mg/kg, i.p) and the improvement of animals' locomotor activities and behavioral changes induced by hyperammonemia. It has been well-known that APAP metabolism in the liver, by CYP2E1, could lead to the production of reactive metabolites (eg, N-acetyl paraquinone imine;

NAPQI) that severely damage cellular targets.[63] On the other hand, oxidative stress is a well-described phenomenon in the liver of APAP-intoxicated animals.[63] Oxidative stress can destruct different cellular components, such as lipids and proteins. Interestingly, the valuable effects of citicoline in the diminishing of the CNS complications related to HE could be due to its direct effects on brain tissue where this chemical encounter oxidative stress and its associated complications (Figure 5). Our result revealed that citicoline ameliorated symptoms of encephalopathy and led to an increase in the concentrations of all antioxidant marker levels as well as brain tissue antioxidant capacity (Figure 5). These results are in line with the results of a recent study which indicate the antioxidant properties of citicoline as a major mechanism involved in its neuroprotective properties.[64]

As type A of HE is an acute syndrome, we used high doses of citicoline in the current study. Citicoline is a safe pharmaceutical that is used at high doses in humans (eg, in stroke) or experimental models.[65,66] Therefore, high doses of this drug could be administered in acute situations such as HE and hyperammonemia to prevent brain injury. On the other hand, administration of this drug (eg, at lower doses) in

chronic conditions such as sub-chronic HE (Type C) could be the subject of future investigations.

The result of this study revealed that citicoline supplementation (500 mg/kg, i.p) improved animal's locomotor activity, and alleviated biomarkers of oxidative stress in the brain tissue. This study recommends citicoline as a possible protective mediator with therapeutic and antioxidant capability against acute liver failure and HE. Therefore, this drug might be a candidate for attenuating HE adverse effects in clinical settings. However, in the present study, the time of citicoline administration was short. We propose a longer period of citicoline administration in other models of HE. On the other hand, finding the precise mechanisms of citicoline neuroprotective effects warranted further investigations.

Abbreviations

HE, hepatic encephalopathy; APAP, acetyl-para-aminophenol; acetaminophen; GSH, glutathione; ROS, reactive oxygen species.

Acknowledgments

Disclosure

The authors declare no conflicts of interest in this work.

References

1. Hadjihambi A, Arias N, Sheikh M, Jalan R. Hepatic encephalopathy: a critical current review. *Hepatol Int*. 2018;12(1):135–147. doi:10.1007/s12072-017-9812-3

2. Heidari R, Jamshidzadeh A, Ommati MM, et al. Ammonia-induced mitochondrial impairment is intensified by manganese co-exposure: relevance to the management of subclinical hepatic encephalopathy and cirrhosis-associated brain injury. *Clin exp hepatol*. 2019;5(2):109–117. doi:10.5114/ceh.2019.85071

3. Heidari R. Brain mitochondria as potential therapeutic targets for managing hepatic encephalopathy. *Life Sci*. 2019;218:65–80. doi:10.1016/j.lfs.2018.12.030

4. Ferenci P. Hepatic encephalopathy. *Gastroenterol Rep*. 2017;5(2):138–147. doi:10.1093/gastro/gox013

5. Musgrave H, Hilsabeck RC. Hepatic encephalopathy. In: Ravdin LD, Katzen HL (eds.), *Handbook on the Neuropsychology of Aging and Dementia*. Springer; 2019:689–710.

6. Hassan A, Tsuda Y, Asai A, et al. Effects of oral l-carnitine on liver functions after transarterial chemoembolization in intermediate-stage hcc patients. *Mediators Inflamm*. 2015;2015.

7. Sakai Y, Nishikawa H, Enomoto H, et al. Effect of l-carnitine in patients with liver cirrhosis on energy metabolism using indirect calorimetry: a pilot study. *J Clin Med Res*. 2016;8(12):863. doi:10.14740/jocmr2734w

8. Saeed RMA, Ahmed HH, Saleh AAS, Ahmed YS. Curative role of lactulose, l-carnitine, alpha-lipoic acid and combination of l-carnitine and alpha-lipoic acid in a rat model of acute hepatic encephalopathy: biochemical observations. *Trop J Pharm Res*. 2017;16(9):2161–2168. doi:10.4314/tjpr.v16i9.17

9. Jamshidzadeh A, Niknahad H, Heidari R, Zarei M, Ommati MM, Khodaei F. Carnosine protects brain mitochondria under hyperammonemic conditions: relevance to hepatic encephalopathy treatment. *Pharma Nutri*. 2017;5(2):58–63. doi:10.1016/j.phanu.2017.02.004

10. Milewski K, Hilgier W, Fręśko I, et al. Carnosine reduces oxidative stress and reverses attenuation of righting and postural reflexes in rats with thioacetamide-induced liver failure. *Neurochem Res*. 2016;41(1–2):376–384. doi:10.1007/s11064-015-1821-9

11. Chepkova AN, Sergeeva OA, Haas HL. Taurine rescues hippocampal long-term potentiation from ammonia-induced impairment. *Neurobiol Dis*. 2006;23(3):512–521. doi:10.1016/j.nbd.2006.04.006

12. Boer LA, Panatto JP, Fagundes DA, et al. Inhibition of mitochondrial respiratory chain in the brain of rats after hepatic failure induced by carbon tetrachloride is reversed by antioxidants. *Brain Res Bull*. 2009;80(1–2):75–78. doi:10.1016/j.brainresbull.2009.04.009

13. Bémeur C, Vaquero J, Desjardins P, Butterworth RF. N-acetylcysteine attenuates cerebral complications of non-acetaminophen-induced acute liver failure in mice: antioxidant and anti-inflammatory mechanisms. *Metab Brain Dis*. 2010;25(2):241–249. doi:10.1007/s11011-010-9201-2

14. Ommati MM, Jamshidzadeh A, Niknahad H, et al. N-acetylcysteine treatment blunts liver failure-associated impairment of locomotor activity. *Pharm Nutri*. 2017;5(4):141–147. doi:10.1016/j.phanu.2017.10.003

15. Secades J. Citicoline in the treatment of cognitive impairment. *J Neurol Exp Neurosci*. 2019;5(1):14–26. doi:10.17756/jnen.2019-047

16. Secades JJ, Alvarez-Sabin J, Castillo J, et al. Citicoline for acute ischemic stroke: a systematic review and formal meta-analysis of randomized, double-blind, and placebo-controlled trials. *J Stroke Cerebrovasc Dis*. 2016;25(8):1984–1996. doi:10.1016/j.jstrokecerebrovasdis.2016.04.010

17. Grieb P. Neuroprotective properties of citicoline: facts, doubts and unresolved issues. *CNS Drugs*. 2014;28(3):185–193.

18. Iulia C, Ruxandra T, Costin L-B, Liliana-Mary V. Citicoline–a neuroprotector with proven effects on glaucomatous disease. *Romanian j Ophthalmol*. 2017;61(3):152. doi:10.22336/rjo.2017.29

19. Roohi-Azizi M, Arabzadeh S, Amidfar M, et al. Citicoline combination therapy for major depressive disorder: a randomized, double-blind, placebo-controlled trial. *Clin Neuropharmacol*. 2017;40(1):1–5. doi:10.1097/WNF.0000000000000185

20. Yu H, Qing H, Lei Z. Cytidine diphosphate choline improves the outcome of cardiac arrest vs epinephrine in rat model. *Am J Emerg Med*. 2013;31(7):1022–1028. doi:10.1016/j.ajem.2013.03.018

21. Shabrang B, Jamshidzadeh A, Farjam M, Ebrahimpour A, Koohi-Hosseinabadi O. Concurrent assessment of calpain and caspase3 activities in brains of mice with acetaminophen-induced acute hepatic encephalopathy. *Metab Brain Dis*. 2017;32(6):2139–2142. doi:10.1007/s11011-017-0096-z

22. Carter RJ, Morton J, Dunnett SB. Motor coordination and balance in rodents. *Curr Protocols Neurosci*. 2001;15(1):8.12.1–8. 4. doi:10.1002/0471142301.ns0812s15

23. Metz GA, Merkler D, Dietz V, Schwab ME, Fouad K. Efficient testing of motor function in spinal cord injured rats. *Brain Res*. 2000;883(2):165–177. doi:10.1016/S0006-8993(00)02778-5

24. Heidari R, Jamshidzadeh A, Niknahad H, et al. Effect of taurine on chronic and acute liver injury: focus on blood and brain ammonia. *Toxicol Rep*. 2016;3:870–879. doi:10.1016/j.toxrep.2016.04.002

25. Ommati MM, Heidari R, Ghanbarinejad V, Aminian A, Abdoli N, Niknahad H. The neuroprotective properties of carnosine in a mouse model of manganism is mediated via mitochondria regulating and antioxidative mechanisms. *Nutr Neurosci*. 2019;:1–13. doi:10.1080/1028415X.2018.1552399

26. Ommati MM, Heidari R, Ghanbarinejad V, Abdoli N, Niknahad H. Taurine treatment provides neuroprotection in a mouse model of manganism. *Biol Trace Elem Res*. 2019;190(2):384–395. doi:10.1007/s12011-018-1552-2

27. Heidari R, Moezi L, Asadi B, Ommati MM, Azarpira N. Hepatoprotective effect of boldine in a bile duct ligated rat model of cholestasis/cirrhosis. *PharmaNutrition*. 2017;5(3):109–117. doi:10.1016/j.phanu.2017.07.001

28. Jamshidzadeh A, Heidari R, Latifpour Z, et al. Carnosine ameliorates liver fibrosis and hyperammonemia in cirrhotic rats. *Clin Res Hepatol Gastroenterol*. 2017;41(4):424–434. doi:10.1016/j.clinre.2016.12.010

29. Gupta R, Dubey D, Kannan G, Flora S. Concomitant administration of moringa oleifera seed powder in the remediation of arsenic-induced oxidative stress in mouse. *Cell Biol Int*. 2007;31(1):44–56. doi:10.1016/j.cellbi.2006.09.007

30. Socci D, Bjugstad K, Jones H, Pattisapu J, Arendash G. Evidence that oxidative stress is associated with the pathophysiology of inherited hydrocephalus in the h-tx rat model. *Exp Neurol*. 1999;155(1):109–117. doi:10.1006/exnr.1998.6969

31. Ommati MM, Farshad O, Niknahad H, et al. Cholestasis-associated reproductive toxicity in male and female rats: the fundamental role of mitochondrial impairment and oxidative stress. *Toxicol Lett*. 2019;316:60–72. doi:10.1016/j.toxlet.2019.09.009

32. Heidari R, Behnamrad S, Khodami Z, Ommati MM, Azarpira N, Vazin A. The nephroprotective properties of taurine in colistin-treated mice is mediated through the regulation of mitochondrial function and mitigation of oxidative stress. *Biomed Pharmacother*. 2019;109:103–111. doi:10.1016/j.biopha.2018.10.093

33. Heidari R, Ahmadi A, Mohammadi H, Ommati MM, Azarpira N, Niknahad H. Mitochondrial dysfunction and oxidative stress are involved in the mechanism of methotrexate-induced renal injury and electrolytes imbalance. *Biomed Pharmacother*. 2018;107:834–840. doi:10.1016/j.biopha.2018.08.050

34. Ommati MM, Jamshidzadeh A, Heidari R, et al. Carnosine and histidine supplementation blunt lead-induced reproductive toxicity through antioxidative and mitochondria-dependent mechanisms. *Biol Trace Elem Res*. 2019;187(1):151–162. doi:10.1007/s12011-018-1358-2

35. Karamikhah R, Jamshidzadeh A, Azarpira N, Saeedi A, Heidari R. Propylthiouracil-induced liver injury in mice and the protective role of taurine. *Pharm Sci*. 2015;21(2):94–101. doi:10.15171/PS.2015.23

36. Heidari R, Babaei H, Roshangar L, Eghbal MA. Effects of enzyme induction and/or glutathione depletion on methimazole-induced hepatotoxicity in mice and the protective role of n-acetylcysteine. *Advan Pharm Bull*. 2014;4(1):21.

37. Benzie IF, Strain JJ. The ferric reducing ability of plasma (frap) as a measure of "antioxidant power": the frap assay. *Anal Biochem*. 1996;239(1):70–76. doi:10.1006/abio.1996.0292

38. Ozgen M, Reese RN, Tulio AZ, Scheerens JC, Miller AR. Modified 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (abts) method to measure antioxidant capacity of selected small fruits and comparison to ferric reducing antioxidant power (frap) and 2, 2 '-diphenyl-1-picrylhydrazyl (dpph) methods. *J Agric Food Chem*. 2006;54(4):1151–1157. doi:10.1021/jf051960d

39. Shafiekhani M, Ommati MM, Azarpira N, Heidari R, Salarian AA. Glycine supplementation mitigates lead-induced renal injury in mice. *J Exp Pharmacol*. 2019;11:15–22. doi:10.2147/JEP.S190846

40. Butterworth RF. Hepatic encephalopathy. *The Liver*. 2020;615–629.

41. Lima LCD, Miranda AS, Ferreira RN, Rachid MA, ACS E Silva. Hepatic encephalopathy: lessons from preclinical studies. *World J Hepatol*. 2019;11(2):173. doi:10.4254/wjh.v11.i2.173

42. Butterworth RF, Norenberg MD, Felipo V, et al. Experimental models of hepatic encephalopathy: ishen guidelines. *Liver Int*. 2009;29(6):783–788. doi:10.1111/j.1478-3231.2009.02034.x

43. Ebadollahi Natanzi AR, Mahmoudian S, Minaeie B, Sabzevari O. Hepatoprotective activity of phloretin and hydroxychalcones against acetaminophen induced hepatotoxicity in mice. *Iran J Pharm Sci*. 2011;7(2):89–97.

44. Görg B, Karababa A, Schütz E, et al. O-glcnacylation-dependent upregulation of ho1 triggers ammonia-induced oxidative stress and senescence in hepatic encephalopathy. *J Hepatol*. 2019;71(5):930–941. doi:10.1016/j.jhep.2019.06.020

45. Zacharias HD, Zacharias AP, Gluud LL, Morgan MY. Pharmacotherapies that specifically target ammonia for the prevention and treatment of hepatic encephalopathy in adults with cirrhosis. *Cochrane Database Syst Rev*. 2019;6.

46. Griffin JW, Bradshaw PC. Effects of a high protein diet and liver disease in an in silico model of human ammonia metabolism. *Theor Biol Med Model*. 2019;16(1):11. doi:10.1186/s12976-019-0109-1

47. Saleem TH, Shehata GA, Toghan R, et al. Assessments of amino acids, ammonia and oxidative stress among cohort of egyptian autistic children: correlations with electroencephalogram and disease severity. *Neuropsychiatr Dis Treat*. 2020;16:11. doi:10.2147/NDT.S233105

48. Shah SWA, Chen J, Han Q, Xu Y, Ishfaq M, Teng X. Ammonia inhalation impaired immune function and mitochondrial integrity in the broilers bursa of fabricius: implication of oxidative stress and apoptosis. *Ecotoxicol Environ Saf*. 2020;190:110078. doi:10.1016/j.ecoenv.2019.110078

49. Heidari R. The footprints of mitochondrial impairment and cellular energy crisis in the pathogenesis of xenobiotics-induced nephrotoxicity, serum electrolytes imbalance, and fanconi's syndrome: a comprehensive review. *Toxicology*. 2019;423:1–31. doi:10.1016/j.tox.2019.05.002

50. Reddy PVB, Murthy CR, Reddanna P. Fulminant hepatic failure induced oxidative stress in nonsynaptic mitochondria of cerebral cortex in rats. *Neurosci Lett*. 2004;368(1):15–20. doi:10.1016/j.neulet.2004.06.046

51. Fadillioglu E, Gursul C, Iraz M. Effects of caffeic acid phenethyl ester on thioacetamide-induced hepatic encephalopathy in rats. *Prog Neuropsychopharmacol Biol Psychiatry*. 2010;34(8):1440–1445. doi:10.1016/j.pnpbp.2010.07.034

52. Shawcross DL, Shabbir SS, Taylor NJ, Hughes RD. Ammonia and the neutrophil in the pathogenesis of hepatic encephalopathy in cirrhosis. *Hepatology*. 2010;51(3):1062–1069. doi:10.1002/hep.23367

53. Norenberg MD, Baker L, Norenberg LOB, Blicharska J, Bruce-Gregorios JH, Neary JT. Ammonia-induced astrocyte swelling in primary culture. *Neurochem Res*. 1991;16(7):833–836. doi:10.1007/BF00965694

54. Bobermin LD, Wartchow KM, Flores MP, Leite MC, Quincozes-Santos A, Gonçalves C-A. Ammonia-induced oxidative damage in neurons is prevented by resveratrol and lipoic acid with participation of heme oxygenase 1. *NeuroToxicology*. 2015;49:28–35. doi:10.1016/j.neuro.2015.05.005

55. Norenberg MD, Jayakumar A, Rao KR. Oxidative stress in the pathogenesis of hepatic encephalopathy. *Metab Brain Dis*. 2004;19(3–4):313–329.

56. Malaguarnera M, Pistone G, Astuto M, et al. L-carnitine in the treatment of mild or moderate hepatic encephalopathy. *Digest Dis*. 2003;21(3):271–275. doi:10.1159/000073347

57. Grieb P. Citicoline and eye health. In: *Handbook of Nutrition, Diet, and the Eye*. Elsevier; 2019:585–603.

58. Zafonte R. Lessons learned from the citicoline brain treatment trial (cobrit). *Ann Phys Rehabil Med*. 2018;61:e40. doi:10.1016/j.rehab.2018.05.089

59. Adibhatla RM, Hatcher J, Dempsey R. Citicoline: mechanisms and stroke clinical trials. *Neurology*. 2019.

60. Cinar E, Yuce B, Aslan F, Erbakan G. Effect of neuroprotective citicoline eye drops on macular microcirculation. *Int Ophthalmol*. 2020;40(9):2237–2246. doi:10.1007/s10792-020-01404-6

61. Cinar E, Yuce B, Aslan F, Erbakan G. Neuroprotective effect of citicoline eye drops on corneal sensitivity after lasik. *J Refract Surg*. 2019;35(12):764–770. doi:10.3928/1081597X-20191021-01

62. Shaffie N, Shabana ME. Role of citicoline as a protective agent on toluene-induced toxicity in rats. *J Arab Soc Med Res*. 2019;14(1):14. doi:10.4103/jasmr.jasmr_9_19

63. Yan M, Huo Y, Yin S, Hu H. Mechanisms of acetaminophen-induced liver injury and its implications for therapeutic interventions. *Redox Biol*. 2018;17:274–283. doi:10.1016/j.redox.2018.04.019

64. Aminzadeh A, Salarinejad A. Citicoline protects against lead-induced oxidative injury in neuronal pc12 cells. *Biochem Cell Biol*. 2019;97 (6):715–721. doi:10.1139/bcb-2018-0218

65. Safavi M, Hosseini-Sharifabad A, Seyed-Yousefi Y, Rabbani M. Protective effects of citicoline and benfotiamine each alone and in combination on streptozotocin-induced memory impairment in mice. *Clin Psychopharmacol Neurosci*. 2020;18(1):81–92. doi:10.9758/cpn.2020.18.1.81

66. Secades JJ, Alvarez-Sabín J, Castillo J, et al. Citicoline for acute ischemic stroke: a systematic review and formal meta-analysis of randomized, double-blind, and placebo-controlled trials. *J Stroke Cerebrovasc Dis*. 2016;25(8):1984–1996. doi:10.1016/j.jstrokecerebrovasdis.2016.04.010