The Potential Neuroprotective Role of Citicoline in Hepatic Encephalopathy

This article was published in the following Dove Press journal:
Journal of Experimental Pharmacology

**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.
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.5–14

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 (Na2HPO4), sodium phosphate monobasic (NaH2PO4), and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ammonium chloride, Trichloroacetic acid (TCA), and hydroxymethyl amino-methane 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 3 mL 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 KHCO₃: 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 (Ultraspec 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                 | + + +         | + + +              | +            | +                    |
| Citicoline-treated group A  | 134±28 b  | 207±64 b  | 0.18±0.02                 | -            | +                  | +            | +                    |
| Citicoline-treated group B  | 78±11 b   | 147±23 b  | 0.16±0.04                 | +            | +                  | -            | -                    |

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 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
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. 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.

The primary neurological impairments in HE include the destruction of psychomotor, neurocognitive deficits, and impaired motor function. 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 (Figure 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.

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.

Increased levels of ammonia could cause severe oxidative stress, tissue inflammation, edema, and increased intracranial pressure. 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. Cellular mitochondrial are also the primary sources of ROS. 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
hyperammonemia could lead to significant impairment of glutathione stores, oxidative stress indices, and locomotive activity alterations. These complications are also mentioned in recent studies. 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.

Previous studies also confirmed the presence of oxidative stress in the cerebral cortex of hyperammonemia and HE animals. It was found that significant oxidative stress occurred in the cerebral cortex, cerebellum, and pons medulla in animals treated with APAP. 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.

Astrocytes are the main target of ammonia neurotoxicity. 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. 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. Antioxidants also have been shown to have valuable effects in experimental models of HE,
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 phosphatidylcholine57 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**

The authors gratefully acknowledge the Pharmaceutical Sciences Research Center and the Vice-Chancellor of Research Affairs of Shiraz University of Medical Sciences for providing technical and financial (Grant 98-01-36-19776) support of the current investigation.

**Disclosure**

The authors declare no conflicts of interest in this work.

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