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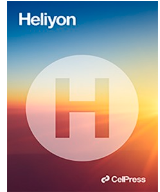

Hepatic encephalopathy complications are diminished by piracetam via the interaction between mitochondrial function, oxidative stress, inflammatory response, and locomotor activity

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A R T I C L E I N F O

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A B S T R A C T

Background: of the study: Hepatic encephalopathy (HE) is a complication in which brain ammonia (NH_4^+) levels reach critically high concentrations because of liver failure. HE could lead to a range of neurological complications from locomotor and behavioral disturbances to coma. Several tactics have been established for subsiding blood and brain NH_4^+. However, there is no precise intervention to mitigate the direct neurological complications of NH_4^+.
Purpose: It has been found that oxidative stress, mitochondrial damage, and neuro-inflammation play a fundamental role in NH_4^+ neurotoxicity. Piracetam is a drug used clinically in neurological complications such as stroke and head trauma. Piracetam could significantly diminish oxidative stress and improve brain mitochondrial function.
Research methods: In the current study, piracetam (100 and 500 mg/kg, oral) was used in a mice model of HE induced by thioacetamide (TA, 800 mg/kg, single dose, i.p).

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Results: Significant disturbances in animals' locomotor activity, along with increased oxidative stress biomarkers, including reactive oxygen species formation, protein carbonylation, lipid peroxidation, depleted tissue glutathione, and decreased antioxidant capacity, were evident in the brain of TA-treated mice. Meanwhile, mitochondrial permeabilization, mitochondrial depolarization, suppression of dehydrogenases activity, and decreased ATP levels were found in the brain of the TA group. The level of pro-inflammatory cytokines was also significantly high in the brain of HE animals.
Conclusion: It was found that piracetam significantly enhanced mice's locomotor activity, blunted oxidative stress biomarkers, decreased inflammatory cytokines, and improved mitochondrial indices in hyperammonemic mice. These data suggest piracetam as a neuroprotective agent which could be repurposed for the management of HE.

1. Introduction

Hepatic encephalopathy (HE) is defined as central nervous system (CNS) injury due to hepatic failure (acute or chronic) [1]. A plethora of investigations were carried out to clarify CNS injury mechanisms in HE [2,3]. Meanwhile, ammonium ion (NH_4^+) remains the main culprit for neurological complications of HE [4]. When the liver fails to metabolize NH_4^+ to urea, the plasma and brain level of this neurotoxic molecule is significantly increased. Increased brain NH_4^+ could lead to various complications, including neural and astrocyte cell death, neuroinflammation, severe oxidative stress, mitochondrial impairment, brain edema, and increased intracranial pressure [5–8]. Moreover, a wide range of behavioral and locomotor disturbances are also attributed to HE [7,9–11]. NH_4^+-induced neurotoxicity could permanently damage the brain and influence patients' quality of life [7,11]. It should be noted that HE could cause coma and patient death if not adequately controlled.

Several mechanisms have been suggested for NH_4^+ neurotoxicity [7]. The induction of oxidative stress is one of the most investigated mechanisms for the neurotoxicity of NH_4^+ [12–15]. Previous studies presented evidence of the crucial role of impaired activity of antioxidant enzymes, ROS formation, lipid peroxidation, and damage of intracellular macromolecules (*e.g.*, proteins and DNA) in the brain tissue in various experimental models of HE [12,15–19]. On the other hand, it has been found that the administration of antioxidants could serve as a potential therapeutic strategy to prevent CNS damage in HE [20–22].

Another important mechanism of NH_4^+-induced neurotoxicity is mediated via the adverse effect of this molecule on mitochondria [7]. It has been repeatedly mentioned that NH_4^+ could cause mitochondrial depolarization, mitochondrial permeabilization, interruption of mitochondrial respiratory complexes activity, and impairment in ATP metabolism in various experimental models [7,23,24]. The brain is a high-energy-demand organ. Therefore, disturbances in mitochondrial functionality by NH_4^+ could lead to an energy crisis and deterioration of brain function [7]. On the other hand, it is well known that mitochondria are the key sources of intracellular ROS [25]. Hence, oxidative stress and mitochondrial impairment are tightly interrelated. It has been reported that NH_4^+ could augment mitochondria-originated ROS production and deteriorate oxidative stress in the brain [7].

Another exciting mechanism of brain injury in HE is mediated through neuro-inflammation [3,26,27]. It has been found that inflammatory cytokines dramatically increased in the brain during HE [3,26,27]. NH_4^+ is suspected of producing pro-inflammatory cytokines from brain microglia or endothelial cells [26]. These cells could release a significant amount of TNF-α and IL- 1β during hyperammonemia [28,29]. The released cytokines could play a crucial role in HE complications such as brain edema [3,28]. Interestingly, there is also evidence of the interplay between HE-associated neuroinflammation, excitotoxicity, and oxidative stress [9,30,31]. In the current study, the level of pro-inflammatory cytokines in the animal model of HE is assessed.

Current therapeutic strategies in HE aims to diminish blood NH_4^+ levels [32]. In this regard, several medications (*e.g.*, lactulose and unabsorbable antibiotics) and dietary interventions (*e.g.*, restricting protein and sodium intake) have been developed for lowering plasma NH_4^+ in HE [32]. However, removing NH_4^+ from the circulation may have no immediate or direct action on NH_4^+-induced neurotoxicity. On the other hand, conditions such as "acute-on-chronic liver failure and HE" and "minimal HE" are linked with chronic hyperammonemia [33,34]. Elevated levels of NH_4^+ in these clinical conditions gradually impair brain function, memory, and cognition [34]. Hence, it is crucial to investigate potential safe and clinically-applicable neuroprotective agents which directly counteract NH_4^+ neurotoxicity. These therapeutic agents could considerably prevent NH_4^+ neurotoxicity until more permanent interventions (*e.g.*, liver transplantation) could be supplied for HE patients.

Although some clinical approaches have been established for blunting plasma ammonia in HE [35], there is no precise strategy for preventing or mitigating neurological complications of HE. Piracetam, a derivative of the neurotransmitter γ-amino butyric acid (GABA), is clinically administered in a variety of neurological complications such as dementia, brain injuries (*e.g.*, trauma), cognitive impairment (*e.g.*, in aging), brain ischemia, epilepsy, and Parkinson's disease [36]. Piracetam is also a metabolic enhancer and "nootropic" drug that profoundly enhances cognitive performance in brain disease [37]. Although there is a long way to go in identifying the precise mechanism of piracetam's neuroprotective effects, several studies mentioned the impact of this drug in blunting brain biomarkers of oxidative stress [38,39]. Many studies also evaluated the effects of piracetam on mitochondrial function [37,40]. It has been found that piracetam could significantly enhance mitochondrial membrane potential, increase ATP levels, avoid mitochondrial swelling, and diminish mitochondria-associated cell death in different experimental models of CNS injury [37,40]. The effect of piracetam on neuro-inflammation is also an interesting characteristic of this drug [41,42]. Piracetam could significantly decrease the release of various types of inflammatory cytokines and blunt their adverse effects on the CNS [41].

As mentioned, oxidative stress, mitochondrial dysfunction, and neuroinflammation are key mechanisms implicated in the neurotoxicity of NH_4^+. Hence, the current study aimed to evaluate the effects of piracetam on locomotor activity, biomarkers of oxidative stress in the brain, inflammatory cytokines levels, and brain mitochondria function in an experimental model of liver failure-induced hyperammonemia and HE.

2. Material and methods

2.1. Reagents

Thiobarbituric acid (TBA), glutathione (GSH), dithiobis-2-nitrobenzoic acid (DTNB), 2′,7′-Dichlorofluorescein diacetate, sodium phosphate monobasic (NaH_2PO_4), trolox, sodium phosphate dibasic (Na_2HPO_4), 2-Oxo-1-pyrrolidine acetamide, potassium chloride (KCl), malondialdehyde, EDTA, HEPES, and 2-(2-Oxopyrrolidino)-acetamide (piracetam), were purchased from Sigma (St. Louis, USA). Sucrose, trichloroacetic acid (TCA), Triton X-100, 2,4-di-nitro phenyl hydrazine (DNPH), MTT, calcium chloride anhydride, rhodamine 123, tetra-butyl ammonium hydroxide, acetonitrile, iron chloride hexahydrate, hydroxymethyl aminomethane hydrochloride (Tris-HCl), EGTA, bovine serum albumin, and TPTZ were purchased from Merck (Darmstadt, KGA, Germany). Commercial kits were used to measure plasma markers of organ injury (Pars-Azmun®, Tehran, Iran). Kits for evaluating pro-inflammatory cytokines were purchased from Jianglai Biology® (Shanghai, China).

2.2. Animals

Mature male C57BL/6 J mice (n = 64, 25 ± 2 g) were obtained from Shiraz University of Medical Sciences, Shiraz, Iran and randomly allocated to four groups (8 animals/group) before starting treatments. Mice were maintained in a standard condition (temperature 23 ± 2 °C, 12: 12 photoschedule, and 42 ± 3% humidity). All procedures were approved by the official ethics board of Shiraz University of Medical Sciences, Shiraz, Iran (Ethics committee code: IR.SUMS.REC.1400.057). The ARRIVE guidelines on animal research were also followed [43].

2.3. Experimental setup

Based on previously reported studies, thioacetamide (TA, 800 mg/kg, i.p, single dose) was used to induce acute liver failure [44]. The TA model is used as an accepted experimental tool to induce hyperammonemia and HE [45]. This model provides acute liver failure and a surge in plasma and brain ammonia [45]. It should be mentioned that some post-TA administration cares including dextrose (2.5 mL/kg of 5% solution, *s.c*) administration should be considered to prevent hypoglycemia, weight loss, renal failure, and animal death [5]. Piracetam (100 and 500 mg/kg, oral) treatment was started 96 h after TA and repeated for three consecutive days (n = 8 animals/groups). The dose of piracetam was within the range of this drug used in previous studies to investigate its neuroprotective properties [36,40]. Seven days after TA administration, animals' locomotor activities were evaluated. Then, animals were anesthetized (90 mg/kg of thiopental, i.p.), and brain and blood specimens were collected for further evaluation. It should be noted that regarding the small brain size of mice, another set of treatments was carried out to evaluate the effect of piracetam on brain mitochondrial indices in hyperammonemic animals.

2.4. Animals' survival rate

Animals (8 mice/group) were monitored for seven days to evaluate animals' survival rates. The probability of animals' survival in each group was analyzed based on the Kaplan-Meier estimate.

2.5. Animals' locomotor activity

Several experimental and clinical evidence signify alterations in locomotor, cognitive, and behavioral activities during HE [45–47]. Cognitive impairment as well as locomotor activity disturbances are evident in patients with chronic or acute forms of HE [48]. Therefore, a series of tests have been developed to assess the effect of therapeutic interventions on ammonia-induced changes in behavioral, cognitive, and locomotor activities [35,45,46,49]. In the current study, a range of tests including rotarod test, open field behavior, gait stride test, negative geotaxis, grip strength test, and beam walk behavior was monitored in hyperammonemic animals to assess potential neuroprotective properties of piracetam.

2.5.1. Rotarod test

The rotarod performance of animals was assessed to evaluate their locomotor function in HE [49,50]. For this purpose, animals were placed on a rotating rod (speed of 5 and 10 rpm; cut-off point = 300 s) [49,50]. Each animal underwent three episodes of rotarod test at least 30-min intervals. The time that animals could stay on the rotarod apparatus was measured [49,50].

2.5.2. Open-field behavior analysis

The open-field analysis was conducted to assess animals' locomotor activity in HE [5]. Briefly, the open-field arena was made of a white Plexiglas box (30 cm L × 30 cm W × 20 cm H). The box floor was alienated into 36 squares (5 × 5 cm). The arena was equipped

with a webcam, and mice activities were evaluated for 15 min per animal. The total number of crossed squares (total locomotion) was recorded [5,51].

2.5.3. *Gait stride activity*

The gait stride activity was used as an index of animals' locomotion in the HE model [5,49]. For this purpose, animals' hind paws were dampened in ink, and mice were let to walk on a 60 cm × 10 cm paper strip covering the same size corridor. The distances (cm) between the right and left hind paws points were measured [49,52].

2.5.4. *Negative geotaxis test*

The negative geotaxis activity was performed as an index of animals' locomotion based on a previously-reported protocol [53]. Briefly, mice were placed on an inclined (30°) surface with their heads facing down [53]. Then, the time to turn 180° was recorded for each animal. The cut-off point for the negative geotaxis test was 90 s [53].

2.5.5. *Grip strength test*

The grip strength of mice's forelimb was measured using a BIOSEB® grip strength meter (BiOSEB®, *in vivo* Research Instruments, USA) based on the device instructions and recorded as an index of locomotor and neurological activity in hyperammonemic mice [54].

2.5.6. *Beam walk test*

Based on a previous protocol, mice were allowed to cross an 80 cm long wooden beam (12 mm diameter; elevated 0.5 m over the ground). A resting box (10 × 10 cm) was designed at one end of the beam. First, animals were trained with three trials (at least three trials with 30-min intervals). Afterward, the time of the beam cross for each animal was measured [53,55].

2.6. *Sample collection*

After locomotor activity tests, mice were anesthetized (90 mg/kg of thiopental, i.p). The blood samples (1000 μL) were collected (abdominal *vena cava*) in standard tubes (KEMICO®, Egypt). Samples were centrifuged (7000 *g*, 15 min, 4 °C), and the plasma was separated and used for biochemical measurements. The whole brain was also excised and used for further research.

2.7. *Plasma biochemistry*

Commercial kits (Parss-Azmuun®, Tehran, Iran) and autoanalyzer (MindrayBS-200®, China) were used to assess liver injury biomarkers in TA-treated mice. A method based on the phenate-hypochlorite reaction was used to assess plasma and brain ammonia levels [5]. For analyzing brain ammonia levels, samples of the brain cortex (100 mg) were treated with 3 mL of 6%, w: v trichloroacetic acid (TCA) solution (4 °C), homogenized, and centrifuged (16000 *g*, 15 min, 4 °C). Then, the supernatant was collected and treated with 100 μL of 2 M $KHCO_3$ (pH = 7) and used for evaluating ammonia levels [5]. Samples of the brain were also homogenized in 40 mM Tris-HCl buffer (pH = 7.4) and centrifuged (17000 *g*, 15 min). The supernatant was used for evaluating pro-inflammatory cytokines. Commercial kits (Jianglai Biology®, Shanghai, China) were used to assess IL-6, TNF-α, and IL-1β levels.

2.8. *Brain non-protein thiols (NPSH) level*

A method based on the colorimetric assessment of the reaction of dithiobis-2-nitrobenzoic acid (DTNB) with non-protein thiols (NPSH) was used [5,56,57]. Briefly, 0.5 mL of the brain tissue homogenate (10% w: v in 40 mM Tris-HCl buffer) was treated with 100 μL of 50% w: v trichloroacetic acid (4 °C) and incubated on ice (5 min). Then, samples were mixed well and centrifuged (10,000 *g*, 4 °C, 20 min). Subsequently, the supernatant was treated with 1000 μL of 40 mM Tris-HCl buffer (pH = 8.9; 4 °C) and 100 μL of DTNB solution (20 mg in 5 mL methanol, protected from light) and mixed well. Finally, the absorbance was measured at λ = 412 nm using an EPOCH® multifunctional plate reader [58].

2.9. *Lipid peroxidation*

The lipid peroxidation level in the brain of hyperammonemic mice was measured based on the thiobarbituric acid reactive substances (TBARS) assay [5,56,59,60]. Briefly, brain samples (250 mg) were homogenized in Tris-HCl buffer (2.5 mL of 40 mM solution, pH = 7.4). Then, 0.5 mL of the resultant tissue homogenate was treated with 0.5 mL of TBA (0.6%, w: v solution) and 0.5 mL of trichloroacetic acid (50% w: v solution) [61–64]. Samples were vortexed (30 s) and incubated in a 100 °C water bath for 40 min. Afterward, 0.5 mL of n-butanol was added, mixed well, and centrifuged (10 min, 10000 *g*, 4 °C). Finally, the absorbance of the upper phase (pink color) was measured at λ = 532 nm [18].

2.10. *Brain tissue reactive oxygen species formation*

Brain reactive oxygen species (ROS) level was estimated using a method based on the 2′, 7′-dichlorofluorescein fluorimetry [65–67]. Briefly, 100 μL of the brain tissue homogenate was mixed with 900 μL of 40 mM Tris-HCl buffer (4 °C, pH = 7.4) and treated with 10 μL of DCF-DA (10 μM concentration) [65,68–70]. Samples were incubated in a 37 °C shaker incubator (10 min, protected from

light). Finally, the samples' fluorescence intensity was evaluated (FLUOstar Omega® multifunctional fluorimeter; $\lambda_{excit} = 485$ nm and $\lambda_{emiss} = 525$ nm) [71,72].

2.11. Brain tissue antioxidant capacity

The ferric-reducing antioxidant power (FRAP) test was used to assess the brain's total antioxidant capacity [73]. Briefly, brain tissue homogenate (100 μL) was added to 900 μL of a freshly-prepared FRAP reagent (20 mM $FeCl_3.6H_2O$, 300 mM acetate buffer, and 10 mM TPTZ; pH = 2). Samples were incubated in a shaker incubator for 5 min (37 °C, protected from light). Finally, samples were centrifuged (16,000 *g*, 5 min), and the absorbance was assessed at $\lambda = 593$ nm [74,75].

2.12. Protein carbonylation

The level of protein carbonylation in the brain was measured based on the reaction of 2, 4-dinitrophenyl hydrazine (DNPH) with oxidized proteins [76–78]. Briefly, 1 mL of brain tissue homogenate was treated with 20 μL of Triton X-100 (final concentration of 0.1%. v: v). Samples were mixed and centrifuged (600 *g*, 4 °C, 20 min). The supernatant was treated with 1 mL of 10 mM DNPH (dissolved in 6 N HCl). Samples were incubated in a shaker incubator (25 °C) for 1 h [76]. Afterward, trichloroacetic acid (500 μL of 20% w: v solution) was added, mixed, and centrifuged (10,000 *g*, 10 min). Then, the pellet was washed thrice with 1000 μL of ethanol: ethyl acetate solution (1:1 v: v mixture) to remove unreacted DNPH. Finally, the pellet was re-dissolved (37 °C water bath) in a guanidine hydrochloride solution (6 M, pH = 2), and the absorbance was assessed at $\lambda = 370$ nm.

2.13. Brain mitochondria isolation

The differential centrifugation method was applied to isolate mice's brain mitochondria [79,80]. For this purpose, the whole brain was excised, washed, and shredded in the mitochondria isolation buffer (1 mM EGTA, 80 mM mannitol, 0.1% w: v of bovine serum albumin, 225 mM sucrose, 2 mM HEPES, pH = 7.4). The minced brain tissue was homogenized in the mitochondria isolation buffer (1:10 v: w; buffer: tissue ratio) and centrifuged (1000 *g*, 20 min, 4 °C). Subsequently, the supernatant was centrifuged at 10000 *g* (4 °C for 20 min) to pellet the mitochondrial fraction. The second centrifugation cycle was repeated three times using the fresh isolation buffer. Finally, the pellet was suspended in the fresh incubation buffer (pH = 7.4, 80 mM mannitol, 2 mM HEPES, and 220 mM sucrose) and used for further experiments [81,82].

2.14. Mitochondrial permeabilization

The light scattering method was used to evaluate mitochondrial permeabilization [82–84]. Briefly, isolated brain mitochondria (100 μL) were added to 900 μL of the pre-warmed (30 °C) swelling buffer (70 mM KCl, 10 mM HEPES, 125 mM sucrose, and pH = 7.2) [85,86]. Calcium (Ca^{2+}, 100 μM) was added to induce mitochondria permeabilization [85]. Then, the absorbance was measured at $\lambda =$ 540 nm for 30 min [59]. The differences between each sample's initial and final absorbance (ΔOD) were reported as an index of mitochondrial swelling [87].

2.15. Mitochondrial ATP level

The level of ATP in isolated brain mitochondria was assessed using a previously-reported HPLC procedure [88]. For this purpose, 1 mL of isolated brain mitochondria was treated with 100 μL of TCA (20% w: v, 4 °C), mixed well, and incubated on ice for 10 min. Then, samples were centrifuged (17000 *g*, 4 °C, 20 min), and the supernatant was treated with KOH (100 μL of 1 M solution, 4 °C) [88]. Then, samples were centrifuged again (17000 *g*, 4 °C, 20 min), and 25 μL of supernatant was injected into an HPLC system. A μBondapak™ C18 column (4.6 × 250 mm) was used as the stationary phase. The mobile phase was made of 100 mM KH_2PO_4 (pH = 7 adjusted with KOH), 1 mM of tetrabutylammonium hydroxide, and acetonitrile (2.5% v: v). The HPLC flow rate was 1 mL/min (UV detector, $\lambda = 254$ nm) [88]. In this method, there was a suitable linear relationship among standard ATP samples at a range of 0–600 nM against its peak area (correlation coefficient = 0.994).

2.16. Mitochondrial dehydrogenase activity

The methyl tetrazolium (MTT) assay was utilized to assess mitochondrial dehydrogenase activity [70,89,90]. Briefly, 0.5 mL of the mitochondrial suspension was treated with 40 μL of 0.4% w: v MTT and incubated at 37 °C for 30 min (protected from light). Subsequently, samples were centrifuged (17000 g, 5 min), and the pellet was dissolved in DMSO (1 mL). Finally, the absorbance was assessed at $\lambda = 570$ nm [73].

2.17. Mitochondrial depolarization

Mitochondrial depolarization was assessed using the cationic fluorescent probe, rhodamine 123 [65,91]. Briefly, samples of isolated brain mitochondria were suspended in the depolarization assay medium (70 mM KCl, 10 mM HEPES, 130 mM sucrose, pH = 7.2) and treated with 20 μL of rhodamine 123 (10 μM concentration) and incubated for 10 min at 37 °C incubator shaker (protected from

light). Samples were centrifuged (17000 *g*, 4 °C, 5 min), and the fluorescence intensity of the supernatant was fluorometrically assessed (FLUOstar ® fluorimeter, Germany) λexcit = 485 nm and λemiss = 525 nm, respectively [92,93].

2.18. Statistical methods

Data are given as mean ± SD. Data comparison was carried out by the one-way ANOVA and Tukey's multiple comparisons as the post-hoc test. A $P < 0.05$ were considered statistically significant.

3. Results

Plasma biochemical analysis of TA-treated mice revealed a significant increase in liver injury biomarkers, including plasma ALT, AST, LDH, and bilirubin (Fig. 1-A). Significant liver histopathological alterations, including necrosis, inflammatory cell infiltration, and steatosis, were also evident in TA-treated mice (Fig. 1-C). These data indicate that liver failure is properly induced in the current animal model. Moreover, ammonia plasma and brain levels were dramatically increased in the TA group (Fig. 1-A). It was found that piracetam (100 and 500 mg/kg) had no significant effect on plasma biomarkers, liver histopathological changes, as well as brain and plasma ammonia levels in the current study (Fig. 1). On the other hand, it was detected that piracetam (100 and 500 mg/kg) significantly enhanced the survival of TA-treated animals (Fig. 1-B).

The assessment of mice's locomotor activity revealed a significant decrease in the open field activity (Fig. 2-A), negative geotaxis (Fig. 2-B), beam crossing (Fig. 2-C), grip strength (Fig. 2-D), decreased time on the rotarod (Fig. 2-E), and disturbances in the gait test (Fig. 2-F) in TA-treated mice (Fig. 2). Moreover, the open field activity (Fig. 2-A), negative geotaxis (Fig. 2-B), beam crossing (Fig. 2-C), grip strength (Fig. 2-D), decreased time on the rotarod (Fig. 2-E), and disturbances in the gait test (Fig. 2-F) were significantly impaired in TA-treated hyperammonemic animals (Fig. 2). It was found that piracetam improved animals' locomotor activity at the doses administered in the current study (Fig. 2). The effect of piracetam on some locomotor activity tests (*e.g.*, gait and open field tests) was more evident at the higher dose of this drug (500 mg/kg) (Fig. 2).

Biomarkers of oxidative stress, including increased ROS formation (Fig. 3-A), lipid peroxidation (Fig. 3-B), decreased NPSH levels (Fig. 3-C), depleted tissue antioxidant capacity (Fig. 3-D), along with protein carbonylation (Fig. 3-E), and were detected in the brain of

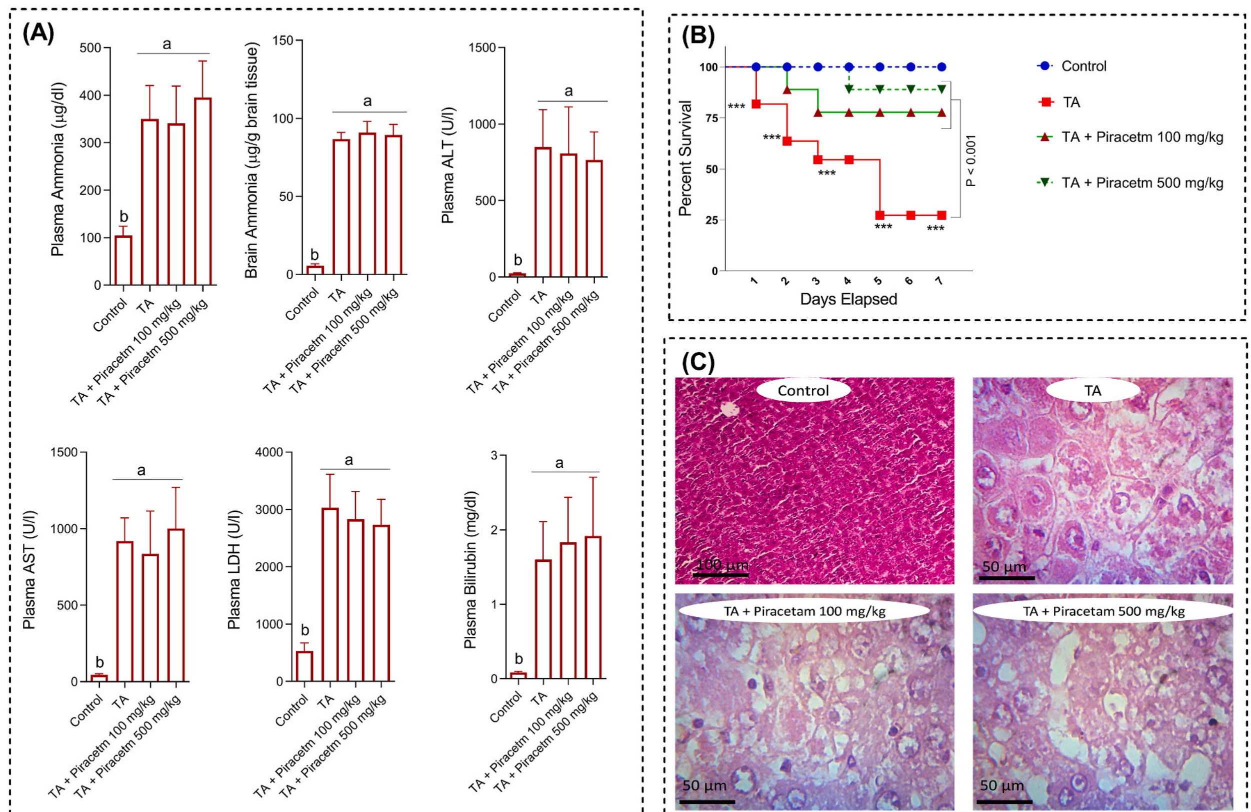

Fig. 1. Plasma biochemistry, plasma ammonia, and brain tissue ammonia levels (A), the survival rate of hyperammonemic mice in the time frame of the current study (B), and liver histopathological alterations in experimental groups (C). TA: Thioacetamide. Data are given as mean ± SD (n = 6). The data sets with different alphabetical superscripts are statistically different ($P < 0.05$).

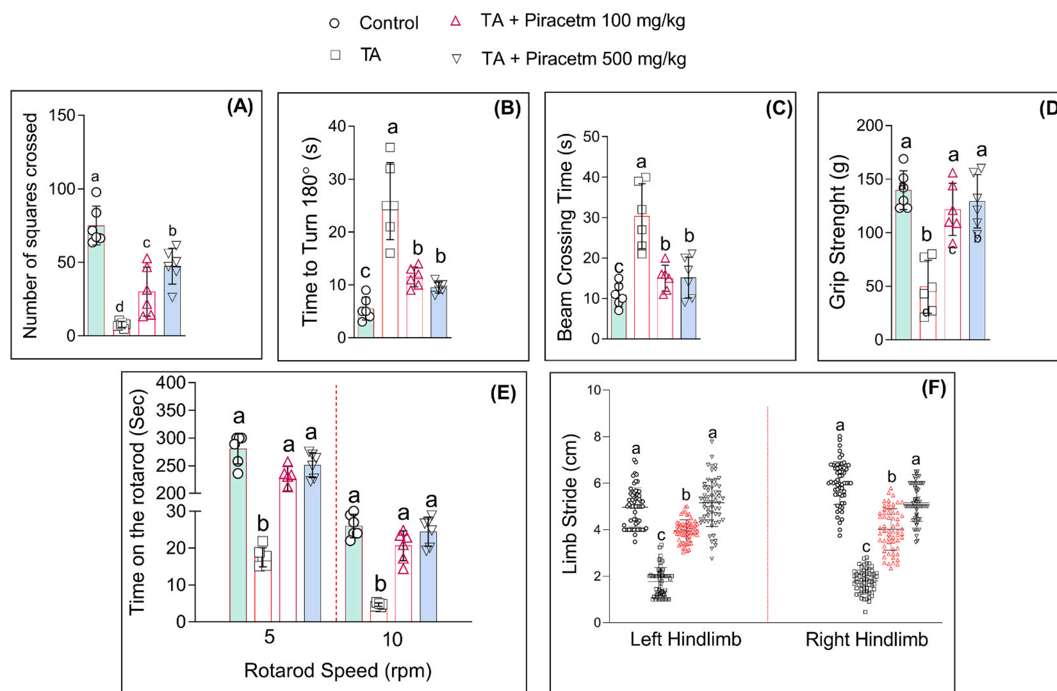

Fig. 2. Mice locomotor activity in thioacetamide (TA)-treated mice. It was found that piracetam significantly improved open field (A), negative geotaxis (B), beam crossing (C), grip strength (D), rotarod (E), and limb stride (F) activities in TA-treated animals. Data are represented as mean ± SD (n = 6). Data for limb stride are shown for at least n = 60. Different alphabetical superscripts indicate a significant difference between groups ($P < 0.05$).

TA-treated animals (Fig. 3). Piracetam (100 and 500 mg/kg) significantly blunted oxidative stress biomarkers in the brain of hyperammonemic mice (Fig. 3). The ameliorative effect of piracetam on brain oxidative stress markers was highlighted in the higher dose (500 mg/kg) in the current model (Fig. 3).

Impaired mitochondrial dehydrogenases activity test (Fig. 4-A), mitochondrial depolarization (Fig. 4-B), mitochondrial permeabilization (4-C), and decreased ATP levels (Fig. 4-D) were the signs of brain mitochondrial impairment in hyperammonemic mice (Fig. 4). It was found that piracetam (100 and 500 mg/kg) significantly improved mitochondrial functionality indices in the brain of hyperammonemic animals (Fig. 4). Similar to the other indices, the positive effects of piracetam on brain mitochondrial indices seemed more evident at the higher dose (500 mg/kg) of this drug (Fig. 4).

Brain level of pro-inflammatory cytokines in including TNF-α (Fig. 5-A), IL-6 (Fig. 5-B), IL-1β (Fig. 5-C) were significantly higher in the TA-treated group (Fig. 5). It was found that piracetam significantly decreased brain levels of inflammatory cytokines in hyperammonemic animals (Fig. 5). No significant difference between the two doses of piracetam was detected in decreasing brain pro-inflammatory cytokines (Fig. 5).

4. Discussion

Hepatic encephalopathy (HE) is an emergency condition that requires instant and restricted clinical interventions. NH_4^+ is the major molecule responsible for neurological complications of HE. Although several strategies have been developed to decrease plasma and brain ammonia in HE patients, there is no particular pharmacological interference to prevent or mitigate the direct neurotoxicity induced by NH_4^+. In the current study, we found that piracetam (100 and 500 mg/kg) could significantly improve locomotor activity, blunt brain oxidative stress biomarkers, mitigate CNS levels of pro-inflammatory cytokines, and enhance brain mitochondrial function in an experimental model of HE. These data could open a new therapeutic window for piracetam as a neuroprotective agent for managing HE complications.

There is strong evidence of the crucial role of oxidative stress and its linked adverse events in HE-associated complications [12,15,31,94,95]. Severe ROS formation, lipid peroxidation, and a decrease in the activity of antioxidant enzymes in the brain tissue have been detected in hyperammonemia experimental models [12,15,31,94,95]. Moreover, several studies revealed the significant therapeutic potential of antioxidant molecules in managing CNS complications of hyperammonemia [92,96–98]. Interestingly, the effect of piracetam on oxidative stress biomarkers have been evaluated in various experimental models of CNS injury *in vitro* and *in vivo* [38,40,99–101]. It has been found that piracetam could enhance the activity of cellular antioxidant systems and contend with severe consequences of ROS formation and oxidative stress, such as lipid peroxidation [38,40]. The precise mechanism involved in the antioxidant properties of piracetam has not been fully understood so far. Some investigations mentioned that the effect of piracetam on

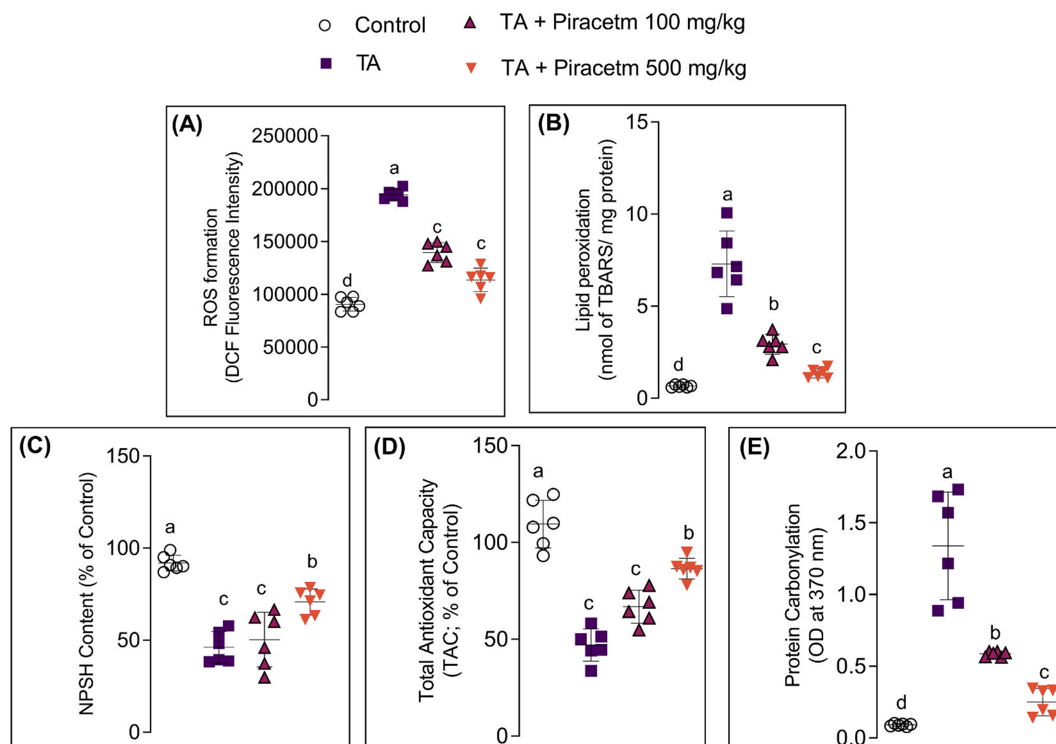

Fig. 3. Oxidative stress biomarkers in the brain of thioacetamide (TA)-treated animals. Piracetam significantly altered oxidative stress biomarkers including ROS formation (A), lipid peroxidation (B), non-protein thiols (NPSH; C), total antioxidant capacity (D), and protein carbonylation (E) in the current study. Data are represented as mean ± SD (n = 6). Different alphabetical superscripts indicate a significant difference ($P < 0.05$).

mitochondria function could play a crucial role in its antioxidative stress in the CNS [40].

On the other hand, as piracetam enhance the expression of cellular antioxidant enzymes [40], the role of this drug in modulating the primary cellular mechanism implicated in the regulation of antioxidant defense mechanisms (*e.g.*, Nrf 2 signaling) [77,102] could be further investigated. In the current study, we found that antioxidants enzymes activity and the level of NPSH were significantly higher in the brain of piracetam-treated hyperammonemic mice (Fig. 3). These data indicate the pivotal role of piracetam's anti-oxidative properties in its neuroprotection mechanisms. It is noteworthy to mention that evaluating the level of molecules such as reduced and oxidized glutathione (GSH and GSSG) and their ratio could give a better estimate of the cellular redox environment. Thus, more studies are warranted to reveal precisely the molecular mechanisms involved in the antioxidant properties of piracetam in the CNS.

Several studies mentioned piracetam as a metabolic enhancer [36,40,103]. These investigations revealed that piracetam could significantly improve ATP levels, recover mitochondrial membrane potential, decrease mitochondria permeabilization, enhance the activity of mitochondrial respiratory chain proteins, and prevent the release of intermediaries of cell death from mitochondria [40, 103]. It has also been found that piracetam could enhance the activity of some mitochondrial matrix-embedded antioxidant enzymes and mitigate mitochondria-mediated ROS formation [103]. Excitingly, the positive effects of piracetam on mitochondrial respiratory proteins activity are also well-established in experimental models of CNS disorders [41]. In the current study, we found that piracetam could significantly improve brain mitochondrial function in hyperammonemic animals by increasing mitochondrial dehydrogenases activity, enhancing mitochondrial polarization, and, most importantly, improving ATP levels (Fig. 4). These data align with previous studies suggesting the positive role of piracetam on brain mitochondrial function [36,40,103]. As mitochondrial damage plays a vital role in the pathogenesis of mitochondrial neurotoxicity and HE complications [7,23,24,92], an essential part of the protective effects of piracetam in the current investigation could be mediated through its positive impact on brain mitochondrial function. Enhancing brain ATP levels and preventing CNS energy crisis by piracetam could also improve animals' locomotor activity observed in the current study (Fig. 2).

Another critical factor involved in the etiology of oxidative stress, neuronal damage, and, finally, locomotor disturbances while HE is the neuro-inflammatory process [3,26,27]. The involvement of inflammatory cytokines released from various cell types or based on different stimuli has been well-studied in experimental models of HE [3,26,27]. Inflammatory response and the release of cytokines are believed to be triggered through various mechanisms [26]. To date, NH_4^+ is known as the primary culprit for the induction of cytokines released in the brain during HE [26]. Mechanisms of NH_4^+-induced increase in pro-inflammatory cytokines in the brain include the stimulation of microglia [26]. Second, some evidence indicates the role of NH_4^+ in targeting inflammatory events in the brain endothelial cells [26]. Pro-inflammatory cytokines released through any mechanism have a final deleterious event which could complicate

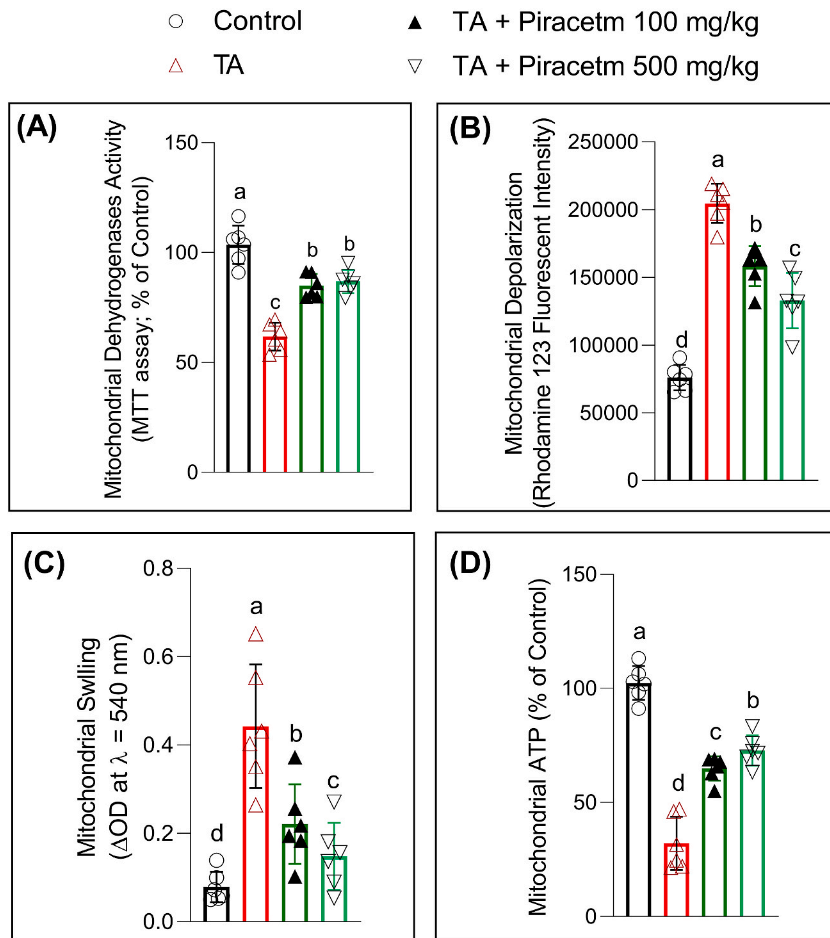

Fig. 4. Piracetam positively altered mitochondrial indices including mitochondrial dehydrogenases activity (A), mitochondrial depolarization (B), mitochondrial swelling (C), and ATP level (D) in the brain of thioacetamide (TA)-treated animals. Data are represented as mean ± SD (n = 6). Columns with different alphabetical superscripts are statistically different (P < 0.05).

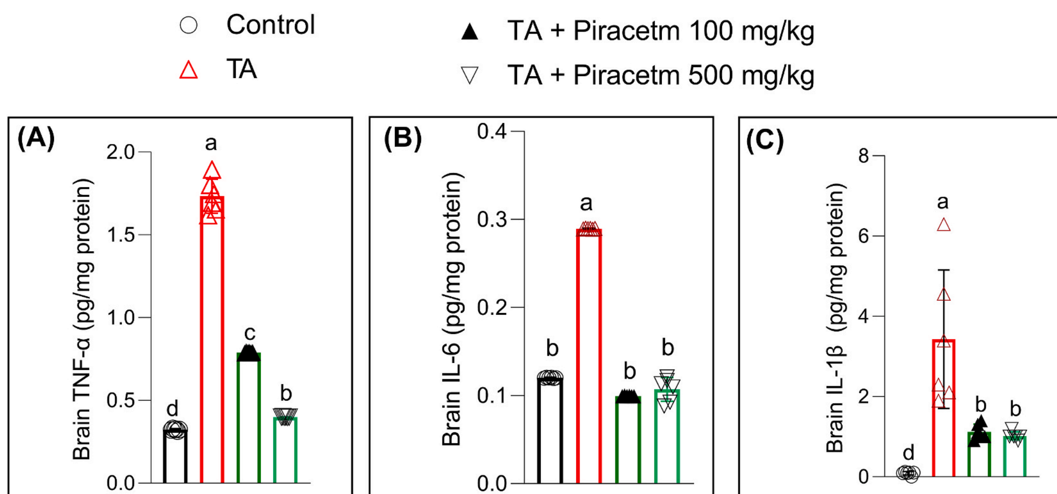

Fig. 5. Pro-inflammatory cytokines in the brain tissue of hyperammonemic mice. Piracetam significantly decreased brain level of TNF-α (A), IL-6 (B), and IL-1β (C) in HE. Data are represented as mean ± SD (n = 6). Columns with different alphabetical superscripts are statistically different (P < 0.05).

the situation of patients with HE. Cytokines influence “astrocytes” in the brain [26]. Astrocytes are involved in brain edema as a lethal complication associated with HE [26]. Brain edema could lead to severe intracranial pressure and even brain herniation [26]. In the current study, we found that piracetam significantly decreased the level of pro-inflammatory cytokines in the brain of hyperammonemic animals (Fig. 5). This effect could play a profound role in the neuroprotective properties of this drug detected in the current study.

Excitingly, the anti-inflammatory effects of piracetam have been repeatedly mentioned in previous studies [41,42]. Tripathi et al. reported that piracetam (50, 100, and 200 mg/kg) significantly decreased neuroinflammation and enhanced brain mitochondrial function and cognitive indices in endotoxin-induced CNS inflammation [41]. Piracetam also has been reported to reduce the level of cytokines (*e.g.*, IL-1β and TNF-α) [42]. Unfortunately, no precise mechanism for the anti-inflammatory properties of piracetam has been described to date. However, an exciting study by Block et al. has reported that levetiracetam, as an analog of piracetam, can significantly block toll-like receptors (TLRs) at very low concentrations [104]. The activation of TLRs in CNS endothelial cells or microglia is pivotal in releasing pro-inflammatory cytokines during HE [26]. Although this effect has not been reported for piracetam to date, it could be a potential mechanism of the anti-inflammatory action of this levetiracetam analog.

An interesting point that should be discussed here is the connection between peripheral benzodiazepine receptors (PBRs) activity in regulating mitochondrial function. Actually, PBRs are proteins located at the outer membrane of mitochondria [95,105]. Excitingly, it has been found that NH_4^+ could induce mitochondrial ROS overload and oxidative stress through PBRs [95,105]. Some in-depth studies suggested PBRs are mitochondrial permeability transition pore (mPT) components [106,107]. On the other hand, it has been found that PBRs are upregulated in HE [106]. It seems that NH_4^+ acts as a PBR agonist and induces mitochondrial permeabilization and oxidative stress. Interestingly, piracetam is an inhibitor of PBRs [108,109]. In the current study, we found that piracetam significantly blunted mPT opening and prevented mitochondrial permeabilization in the brain during HE (Fig. 4). Moreover, mitochondrial membrane potential persevered, and more ATP levels were detected in piracetam-treated hyperammonemic mice (Fig. 4). Therefore, in addition to the effects of piracetam on oxidative stress parameters, its positive role on mitochondrial function could play an essential role in its neuroprotective properties. Meanwhile, evaluating the gene and protein expression levels of PBRs in the piracetam-treated hyperammonemic animals is strongly recommended to fulfill our knowledge about the involved mitochondrial-related routes. Moreover, it is suggested to evaluate brain histopathological changes as well as using techniques such as immunohistochemical analysis of brain tissue to assess the expression of certain proteins in HE and evaluate the effect of therapeutic interventions such as piracetam.

It is noteworthy to mention here that in the current study, we found no significant hepatoprotective properties in piracetam (Fig. 1). This point indicates that the effects of piracetam on mitochondrial function, oxidative stress, and inflammatory response induced by hyperammonemia could be mediated through its direct neuroprotective role in HE. It should also be mentioned that previous studies which evaluated the hepatoprotective properties of piracetam, used different routes of administration, drug doses, and time of treatment in comparison with the current study [110,111].

5. Conclusion

In conclusion, our findings suggested that hyperammonemia caused significant locomotor disturbances, induced oxidative stress in the brain, and impaired brain mitochondrial function. On the other side, piracetam administration offered protection against HE-associated complications. The data obtained from the current study indicate that the effects of piracetam on oxidative stress, neuroinflammation, and mitochondrial function play a vital role in its neuroprotective properties.

Piracetam is a safe drug ($LD_{50} > 10,000$ mg/kg in mouse and rat) that is used at high doses (1.6–9.6 g/day) in diseases such as myoclonus, stroke, trauma, and cerebrocortical insufficiency disorders in human [112]. These findings could utilize the clinical application of piracetam in HE. Therefore, more investigations are warranted to assess the molecular mechanisms of neuroprotection provided by piracetam and assess this drug’s effects on HE-associated neurological complications in clinical settings. Moreover, the effect of various doses of piracetam, its safety in HE, and the effectiveness of this drug in other experimental models of HE could be the subject of further research in this field.

Author contribution statement

R. Heidari, M.M. Ommati, and H. Niknahad, and A. Mobasheri conceived and designed the experiments, analyzed and interpreted the data, wrote the paper, as well as contributed reagents, materials, analysis tools or data. A. Arjmand, E. Rafiei, S. Alidaee, H. Razavi, S. Bagheri, H. Rezaei, S. Sabouri, S. MM. Kashani, A. Najibi, and F. Khodaei performed the experiments and wrote the paper. All authors read, edited, and approved the final version of the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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