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Original paper

Ammonia-induced mitochondrial dysfunction and energy metabolism disturbances in isolated brain and liver mitochondria, and the effect of taurine administration: relevance to hepatic encephalopathy treatment

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Abstract

Introduction: Ammonia-induced oxidative stress, mitochondrial dysfunction, and energy crisis are known as some the major mechanisms of brain injury in hepatic encephalopathy (HE). Hyperammonemia also affects the liver and hepatocytes. Therefore, targeting mitochondria seems to be a therapeutic point of intervention in the treatment of HE. Taurine is an abundant amino acid in the human body. Several biological functions including the mitochondrial protective properties are attributed to this amino acid. The aim of this study is to evaluate the effect of taurine administration on ammonia-induced mitochondrial dysfunction.

Material and methods: Isolated mice liver and brain mitochondria were exposed to different concentrations of ammonia (1, 5, 10, and 20 mM) and taurine (1, 5, and 10 mM), and several mitochondrial indices were assessed.

Results: It was found that ammonia inhibited mitochondrial dehydrogenases activity caused collapse of mitochondrial membrane potential (MMP), induced mitochondrial swelling (MPP), and increased reactive oxygen species (ROS) in isolated liver and brain mitochondria. Furthermore, a significant amount of lipid peroxidation (LPO), along with glutathione (GSH) and ATP depletion, was detected in ammonia exposed mitochondria. Taurine administration (5 and 10 mM) mitigated ammonia-induced mitochondrial dysfunction.

Conclusions: The current investigation demonstrates that taurine is instrumental in preserving brain and liver mitochondrial function in a hyperammonemic environment. The data suggest taurine as a potential protective agent with a therapeutic capability against hepatic encephalopathy and hyperammonemia.

Key words: amino acid, bioenergetics, brain injury, hepatic encephalopathy, taurine.

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Introduction

Hepatic encephalopathy (HE) is a deleterious clinical complication accompanied by acute and chronic liver injury [1]. Although the exact cause of HE is unknown, there is agreement on the predominant role played by ammonia in HE etiology [2]. Ammonia is metabolized by the liver to the urea in healthy subjects. Damaged livers are unable to metabolize ammonia. Hence, this toxic chemical is elevated in the systemic circulation and finally reaches the brain. Ammonia is a neurotoxin that mainly affects astrocytes in the central nervous system (CNS) [3, 4]. It also has several direct toxic effects on neurons [4]. Ammonia causes brain edema, oxidative stress, and inflammation when its level rises in HE [5]. Consequently, a decline in brain function occurs in patients with HE [5]. Hyperammonemia also affects hepatocytes [6].

Disturbed mitochondrial function and oxidative stress are implicated in ammonia-induced cytotoxicity [3, 7]. It has been reported that brain energy metabolism is disturbed in chronic and acute HE [8-10]. Ammonia negatively affects several key enzymes that are responsible for energy metabolism in mitochondria [11]. Hence, the targeting of bioenergetics failure represents a potentially useful approach for the treatment of HE and hyperammonemia disorders.

Taurine is an abundant non-protein amino acid in the human body. The concentrations of taurine in the brain, heart, and muscle are high [12]. Several physiological and pharmacological roles are attributed to taurine [13]. This amino acid is an antioxidant, membrane stabilizer, osmoregulator, and most probably, a neurotransmitter [14-16]. On the other hand, the protective effect of taurine has been investigated against a range of different xenobiotics [17-20]. Taurine has also been shown to have a profound effect on CNS [21, 22]. It has been shown that taurine acts as an osmoregulator, protects neurons, prevents astrocytes swelling, and encounters oxidative stress in CNS [23-26]. The protective effects of taurine against a range of CNS disorders, as well as xenobiotics-induced CNS injury have also been reported [21-26].

The effect of taurine on mitochondria is reported in several investigations [14, 27, 28]. Taurine regulates mitochondrial pH, affects mitochondrial GSH and antioxidants, and preserves the mitochondrial membrane potential [14, 29, 30].

Recently, we found that taurine administration effectively prevented the increase in plasma and brain ammonia after chronic and acute liver failure [31]. The aim of this study is to evaluate the effect of taurine on brain and liver mitochondria as a major target of ammonia-induced toxicity in HE.

Material and methods

Chemicals

4,2-Hydroxyethyl,1-piperazineethanesulfonicacid (HEPES),6-hydroxy-2,5,7,8-tetramethylchroman-2-carbo-2-carboxylic acid (Trolox), 3-(N-morpholino) propane sulfonic acid (MOPS), dimethyl sulfoxide (DMSO), D-mannitol, fatty acid-free bovine serum albumin (BSA) fraction V, Thiobarbituric acid (TBA), 3-[4, 5 dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), Dithiobis-2-nitrobenzoic acid (DTNB), Glutathione (GSH), 2', 7'-Dichlorofluorescein diacetate (DCFH-DA), Taurine, Malondialdehyde (MDA), Sucrose, KCl, Na_2HPO_4, $MgCl_2$, Rhodamine 123 (Rh-123), Coomassie brilliant blue, Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), Sodium succinate, and Ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trichloroacetic acids (TCA), ammonium chloride, and hydroxymethyl amino methane hydrochloride (Tris-HCl) were purchased from Merck (Dardamstd, Germany). All salts for preparing buffer solutions were of analytical grade and prepared from Merck (Dardamstd, Germany).

Animals

Male BALB/c mice (n = 60, 25-30 g) were housed in cages on wood bedding at a temperature of 23 ± 1°C. Animals had free access to food and tap water. The animals received humane care and were handled according to the animal handling protocol approved by a local ethics committee (# 94-01-36-9741).

Mitochondria isolation

Mice liver and brain mitochondria were isolated as previously described [32]. Briefly, animals were anesthetized (Thiopental, 50 mg/kg, i.p.), and their brain and liver were excised, washed and minced in ice-cooled saline (sodium chloride 0.9%) [32, 33]. Mice liver and brain was homogenized in an ice-cooled buffer containing 75 mM mannitol, 220 mM sucrose, 0.5 mM EGTA, 2 mM HEPES, 0.1% BSA (pH = 7.4) at a 10 : 1 buffer to tissue (v/w) ratio [32]. Afterward, tissue homogenate was centrifuged at 1,000 × g for 10 minutes at 4°C to remove intact cell debris and nuclei. The supernatants were further centrifuged at 10,000 × g (4°C for 10 minutes) to precipitate the heavy membrane fractions (mitochondria). The recent step was repeated three times using fresh buffer medium. As mentioned, all manipulations for mitochondria isolation were performed at 4°C or on ice to minimize mitochondrial injury [32].

Ammonia was added with previously reported pathologically relevant concentrations (5 mM, 10 mM) and with a high concentration (20 mM), to the isolated brain and liver mitochondria, and the toxicity markers were monitored [34].

Mitochondrial dehydrogenases activity

The 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was applied as a colorimetric method for determination of mitochondrial functionality in isolated mitochondria [35, 36]. Briefly, mitochondrial suspension in a buffer containing 320 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl

(pH = 7.4), was incubated with 0.4% of MTT at 37°C for 30 minutes. The product of purple formazan crystals was precipitated (10,000 g, 10 min) and dissolved in 1 mL dimethyl sulfoxide (DMSO). Then, 100 µl of dissolved formazan was added to 96 well plate and the optical density (OD) at 570 nm was measured with an EPOCH plate reader (BioTek® Instruments, Highland Park, USA).

Reactive oxygen species (ROS) in isolated mitochondria

The mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA [32]. Briefly, isolated kidney mitochondria were placed in a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM Sodium succinate, and 20 µM Ca^{2+} (pH = 7.2) [32]. Following this step, DCFH-DA was added (final concentration, 10 µM) to mitochondrial suspension, and then incubated for 30 min at 37°C. The fluorescence intensity of the samples was measured using a FLUOstar Omega® multifunctional microplate reader ($\lambda_{excitation}$ = 485 nm and $\lambda_{emission}$ = = 525 nm) [32].

Mitochondrial membrane potential

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential [32]. For this purpose, the mitochondrial fractions (0.5 mg protein/ml) were incubated (30 min, 37°C) with 10 µM of rhodamine 123 in a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 5 mM Sodium succinate, and 20 µM Ca^{2+} (pH = 7.2). Samples were centrifuged (10 min, 10,000 g, 4°C), and the fluorescence intensity of supernatant was monitored using a FLUOstar Omega® multifunctional microplate reader at the excitation and emission wavelength of 485 nm and 525 nm, respectively [32].

Mitochondrial swelling assay

Mitochondrial swelling was measured as previously described [32]. Briefly, the mitochondria were suspended in a buffer containing 125 mM sucrose, 65 mM KCl, 10 mM Hepes-KOH, 20 µM Ca^{2+} (pH = 7.2). Light absorbance at 540 nm was measured at the two-time points (10 and 70 minutes) at 30°C [32]. It is accepted that a decreased light absorbance is consistent with an increase in mitochondrial volume. Hence, as mitochondria are more swelled, the differences between light absorbance of two-time points are higher. The differences between the absorbance of samples were assessed ($\Delta OD^{540\ nm}$) and compared in different experimental groups [32].

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were assessed as an index of lipid peroxidation in isolated liver and brain mitochondria [32]. Briefly, isolated mitochondria were washed to remove sucrose in ice-cold 3-(N-morpholino) propane sulfonic acid (MOPS)-KCl buffer (50 mM MOPS, 100 mM KCl, pH = 7.4), and re-suspended in MOPS-KCl buffer. Afterward, the mitochondrial suspension was mixed with twice its volume of 15% trichloroacetic acid, 0.375% thiobarbituric acid (TBA), 0.24 N HCl plus 0.5 mM Trolox, and heated for 15 min at 100°C [32, 37]. After centrifugation (15,000 g, 1 min), the absorbance of the supernatant was assessed at 532 nm with an Epoch plate reader (BioTek® Instruments, Highland Park, USA) [32, 36].

Mitochondrial glutathione (GSH) content

GSH level was determined with the spectrophotometric method using 5, 50-dithiobis-2-nitrobenzoic acid (DTNB) as an indicator of GSH [32]. The mitochondrial suspension was treated with trichloroacetic acid (10%, w/v) to extract mitochondrial glutathione. The mixture was centrifuged (15,000 g, 4°C, 1 min) to remove denatured proteins [32]. Afterward, 100 µl of DTNB (0.04% in phosphate buffer, pH = 8.9) was added and the intensity of produced color was recorded at 412 nm with an ultraviolet spectrophotometer [36].

Mitochondrial ATP level

A luciferase-luciferin-based kit (ENLITEN®, Promega) was used to assess mitochondrial ATP content [38]. Samples and buffer solutions were prepared based on the kit instructions and the luminescence intensity of samples was measured at 560 nm using a FLUOstar Omega® multifunctional microplate reader.

Samples protein content

For standardization of data, samples protein concentrations were determined by the Bradford method [39].

Statistical analysis

Data are given as the mean ± SD (n = 6). Data comparison was performed by the one-way analysis of

variance (ANOVA) with Tukey's multiple comparison test as a *post hoc*. Differences were considered statistically significant when $p < 0.05$.

Results

The alterations in mitochondrial dehydrogenases activity (MTT test) under the various incubation of NH_4^+ concentrations are summarized in Figure 1. It was found that NH_4^+ concentrations higher than 5 mM significantly decreased mitochondrial dehydrogenases activity in both isolated brain and liver mitochondria. Isolated mitochondria were also incubated with different taurine concentrations. Taurine caused no significant decrease in mitochondrial dehydrogenases activity when it was administered alone. Concentrations of 5 mM and 10 mM of taurine seem to preserve isolated mitochondria in a more functional state as judged by mitochondrial dehydrogenases activity (Fig. 1).

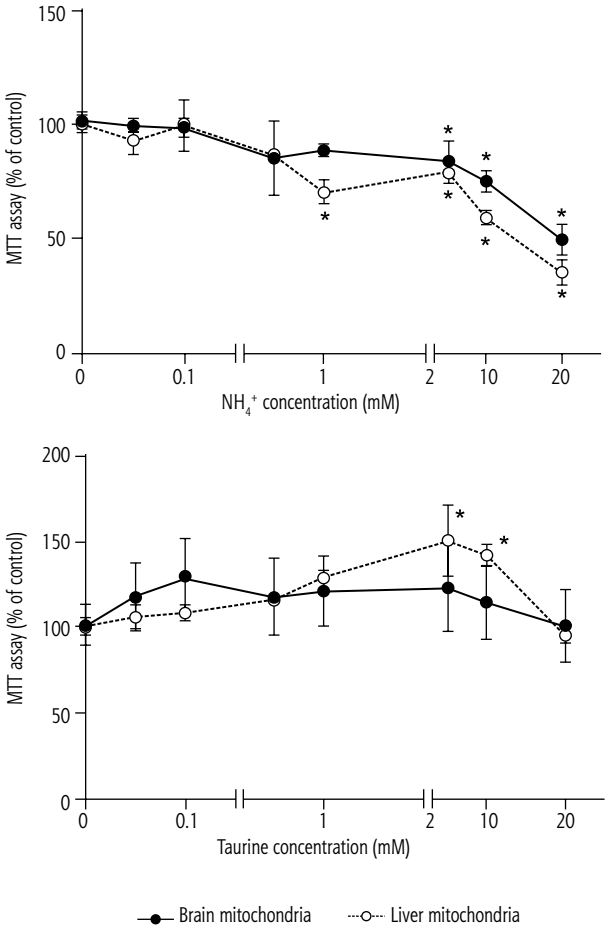

Fig. 1. Concentration-response of ammonia and taurine in isolated mice brain and liver mitochondria. NH_4^+: ammonium ion. Data are given as mean ± SD ($n = 6$). *– Indicates significantly different from concentration 0 mM ($p < 0.05$)

The effect of taurine administration against ammonia-induced mitochondrial dysfunction was investigated. It was found that taurine (5, 10, and 20 mM) significantly prevented the ammonia-induced decrease in mitochondrial dehydrogenases activity in isolated brain and liver mitochondria (Fig. 2).

A significant amount of reactive oxygen species (ROS) was detected in ammonia-treated groups (Fig. 3). On the other hand, taurine (10 mM) effectively mitigated ammonia-induced oxidative stress in brain and liver mitochondria (Fig. 3).

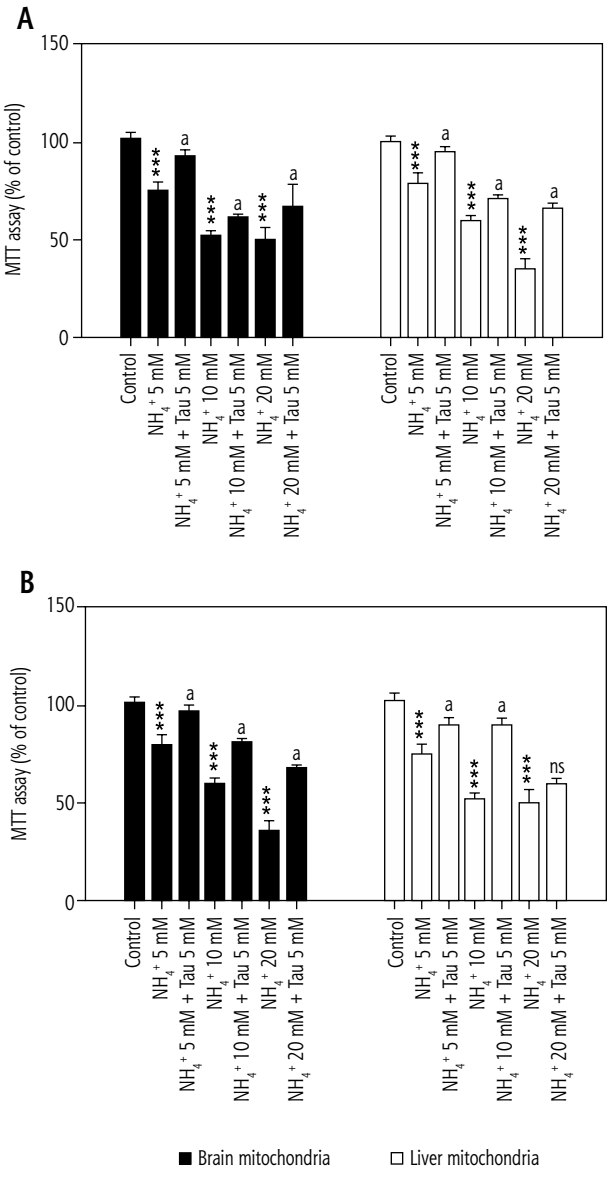

Fig. 2. The effect of taurine administration on the ammonia-induced decrease in mitochondrial succinate dehydrogenase activity. **A)** Taurine 5 mM, **B)** Taurine 10 mM. Tau: taurine, NH_4^+: ammonium ion. Values are represented as mean ± SD ($n = 6$). *** – Indicates significantly different from control ($p < 0.001$). a – Indicates significantly different from ammonia-treated group ($p < 0.05$). ns – Indicates not significant as compared with ammonia-treated group

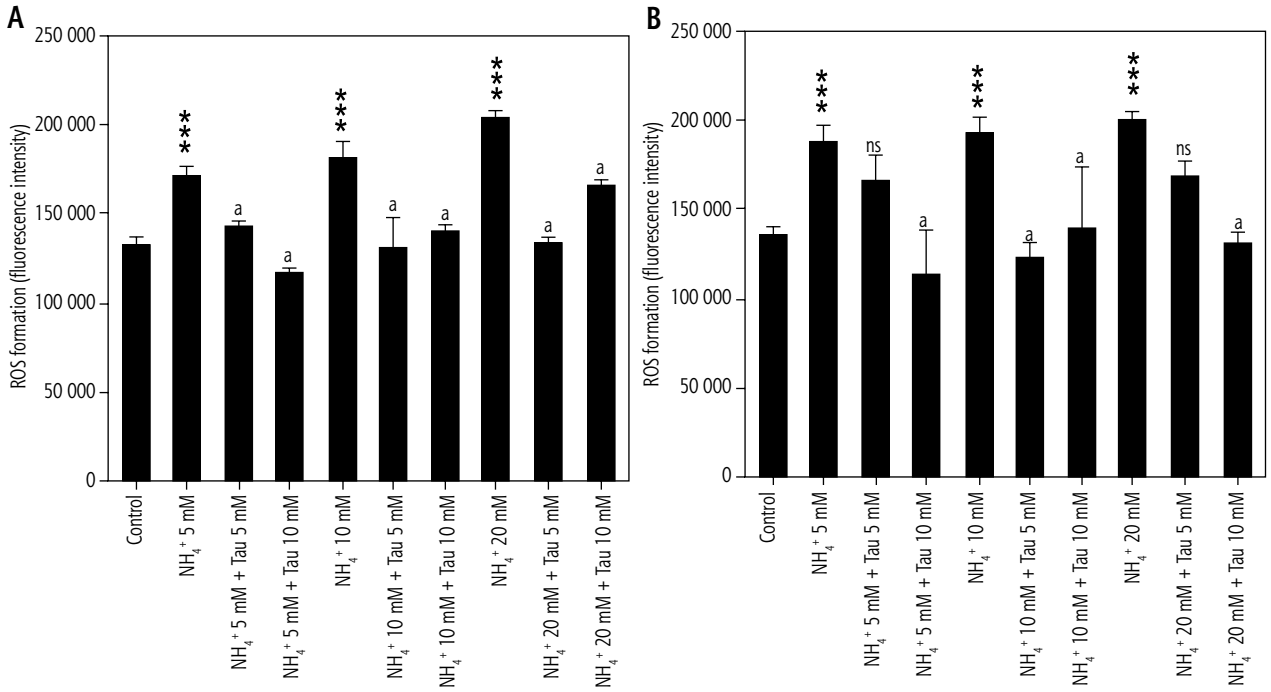

Fig. 3. Effect of taurine on ammonia-induced ROS formation in the isolated brain **(A)** and liver **(B)** mitochondria. Tau: taurine, NH_4^+: ammonium ion. Data are given as mean ± SD (n = 6). *** – Indicates significantly different from control ($p < 0.001$). a – Indicates significantly different from the only ammonium-treated group ($p < 0.001$). ns – not significant as compared with the ammonia-treated group

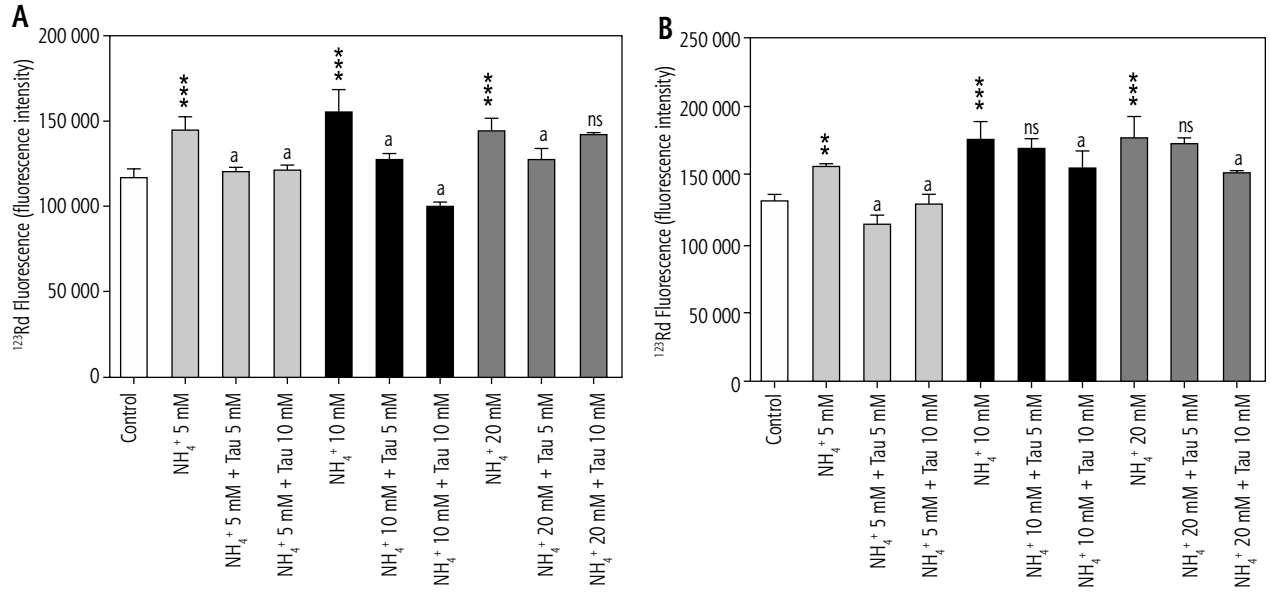

Fig. 4. Ammonia-induced mitochondrial membrane collapse and the effect of taurine. **A)** Isolated brain mitochondria, **B)** isolated liver mitochondria. Tau: taurine, NH_4^+: ammonium ion. Asterisks indicate significantly different as compared with control (**$p < 0.01$, ***$p < 0.001$). a – Indicates significantly different as compared to ammonia-treated group ($p < 0.001$). ns – Indicates not significant as compared with the ammonia-treated group

It was found that ammonia (5, 10, and 20 mM) caused mitochondrial membrane potential ($\Delta\Psi$) collapse (Fig. 4). Also, taurine (10 mM), effectively mitigated the ammonia-induced decrease in mitochondrial membrane potential (Fig. 4).

When different concentrations of ammonia were added to isolated mitochondria, a marked increase of mitochondrial swelling was evident (Fig. 5). It was found that the induction by ammonia of mitochondrial swelling was significantly mitigated by taurine administration (Fig. 5).

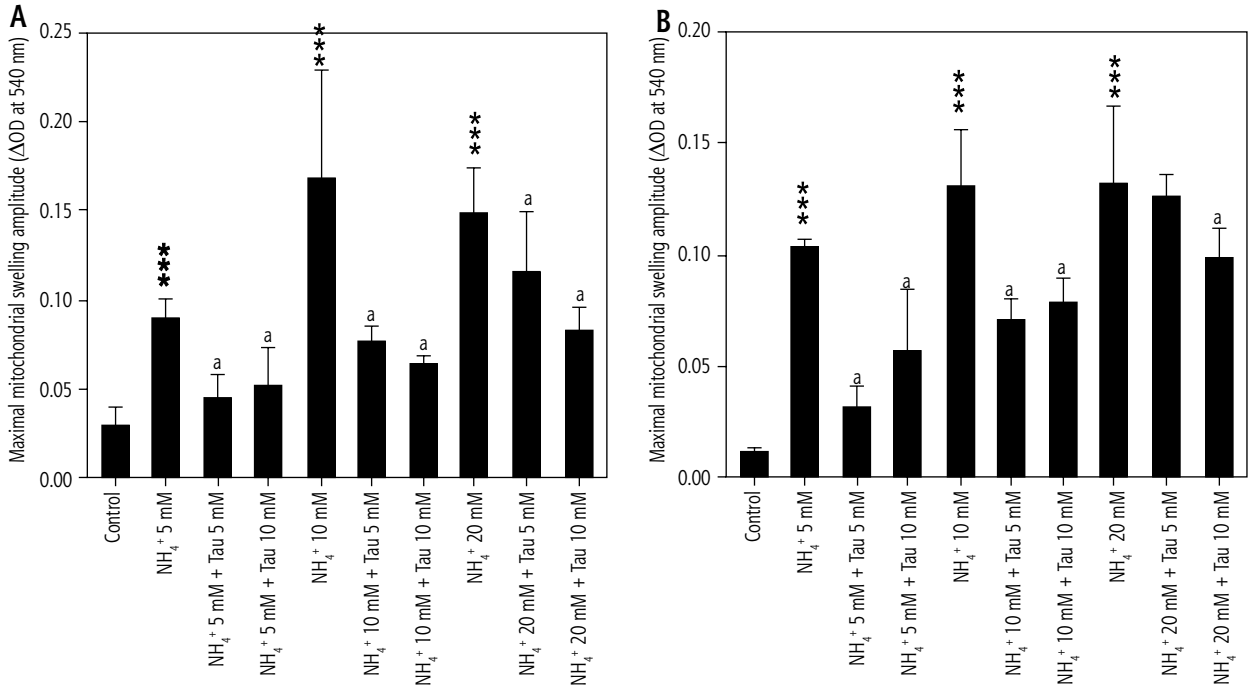

Fig. 5. Effect of taurine on ammonia-induced mitochondrial swelling. **A)** Isolated brain mitochondria, **B)** isolated liver mitochondria. Tau: taurine, NH_4^+: ammonium ion. Data are represented as mean ± SD (n = 6). *** – Indicates significantly different from control ($p < 0.001$). a – Indicates significantly different as compared with the ammonia-treated group ($p < 0.05$). ns – not significant as compared with the ammonia-treated group

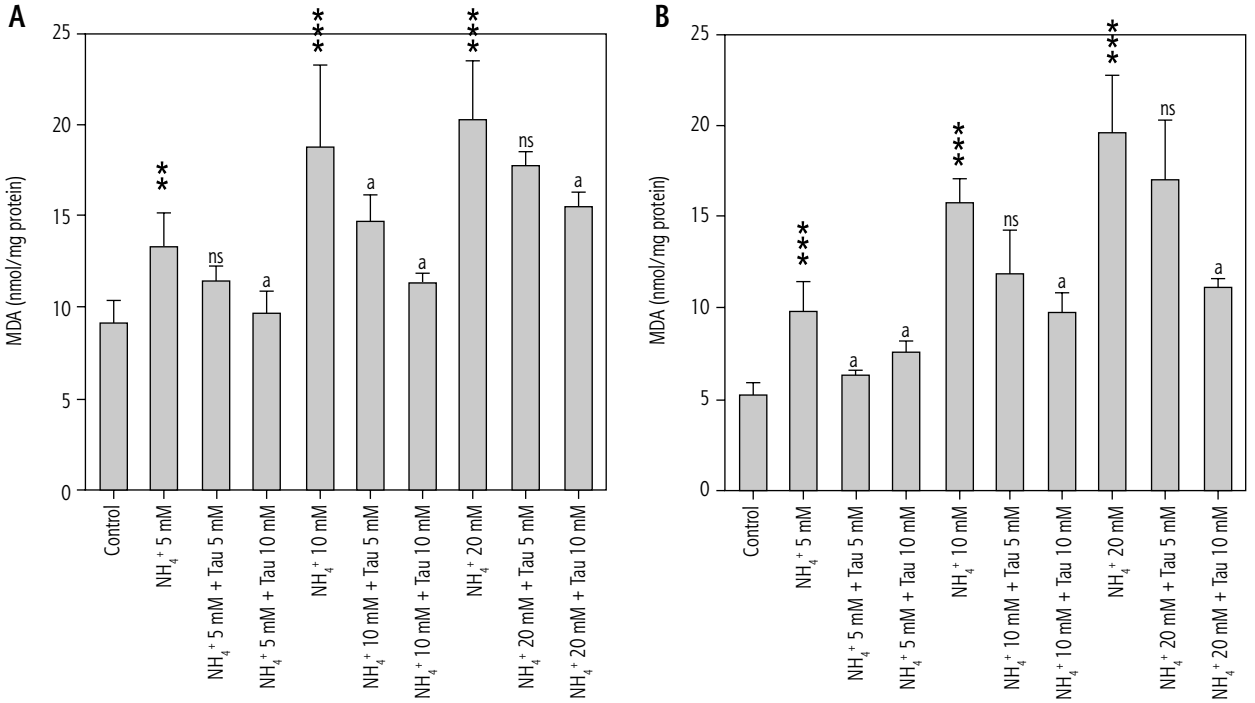

Fig. 6. Ammonia-induced lipid peroxidation in isolated brain **(A)** and liver **(B)** mitochondria. Tau: taurine, NH_4^+: ammonium ion. Values are given as mean ± SD (n = 6). Asterisks indicate significantly different as compared with control (**$p < 0.01$, ***$p < 0.001$). a – Indicates significantly different as compared to the same ammonia concentration-treated group ($p < 0.001$). ns – not significant as compared with the ammonia-treated group

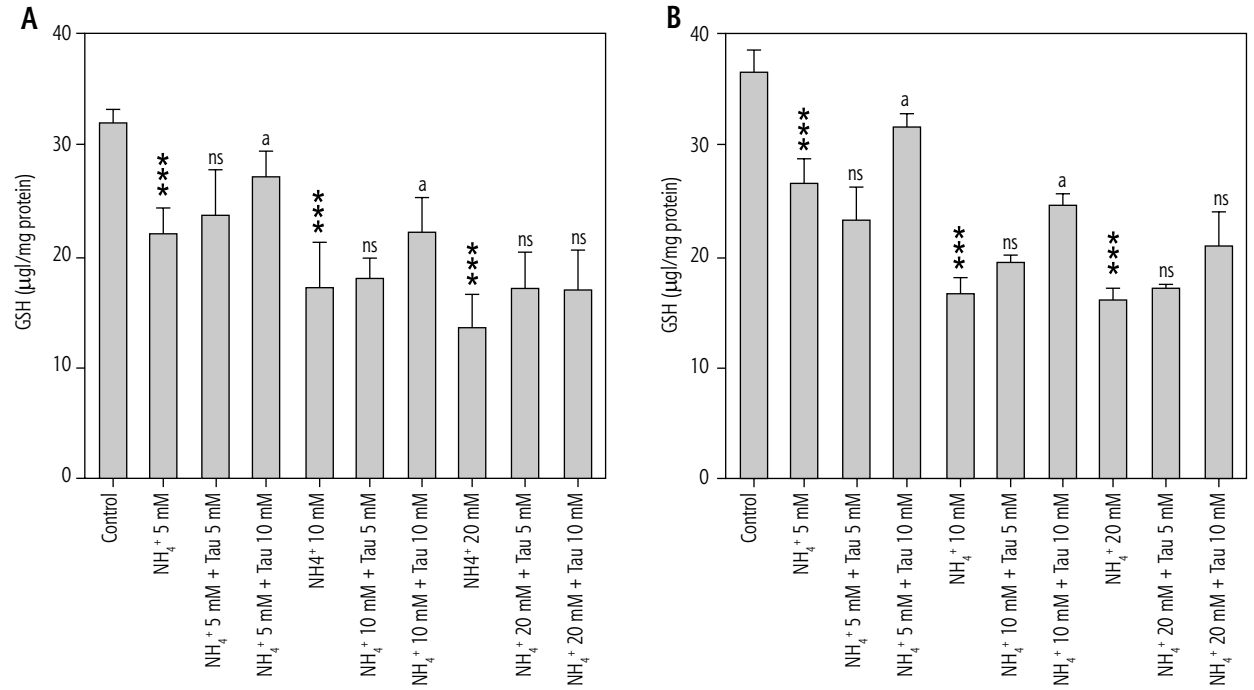

Fig. 7. The effect of ammonia on mitochondrial GSH content. **A)** Isolated brain mitochondria, **B)** isolated liver mitochondria. Tau: taurine, NH_4^+: ammonium ion. Data are represented as mean ± SD (n = 6). *** – Indicates significantly different from control ($p < 0.001$). a – Indicates significantly different from the ammonia-treated group ($p < 0.05$). ns – not significant as compared with the ammonia-treated group

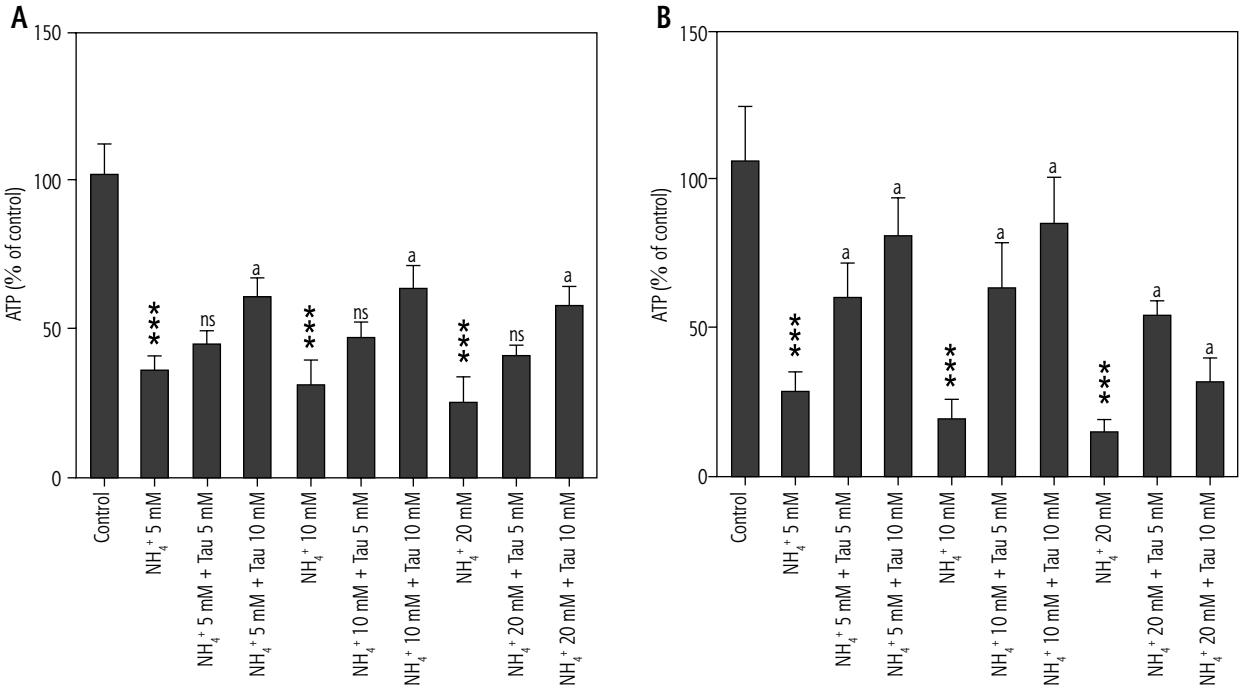

Fig. 8. Mitochondrial ATP level. Tau: taurine, NH_4^+: ammonium ion. **A)** Isolated brain mitochondria, **B)** isolated liver mitochondria. Data are represented as mean ± SD (n = 6). ***– Indicates significantly different from control ($p < 0.001$). a – Indicates significantly different from the ammonia-treated group ($p < 0.05$). ns – not significant as compared with the ammonia-treated group

Ammonia also induced significant lipid peroxidation (Fig. 6) and glutathione and ATP depletion (Figs. 7 and 8). Taurine prevented ammonia-induced lipid peroxidation (Fig. 6), glutathione reservoir depletion (Fig. 7), and a decrease in mitochondrial ATP level (Fig. 8) in isolated brain and liver mitochondria.

Discussion

Ammonia is a neurotoxin underlying the pathogenesis of HE [2, 40]. Mitochondria are major targets of ammonia-induced cytotoxicity [7, 8, 10]. Hence, protecting this organelle may prevent ammonia-induced cellular injury and has clinical benefits in hyperammonemia conditions. In the current investigation, we found that taurine administration effectively preserved isolated brain and liver mitochondrial function in a hyperammonemic environment.

Ammonia-induced oxidative/nitrosative stress in CNS is accompanied by extensive lipid peroxidation and cellular glutathione reservoirs depletion [41, 42]. We found that ammonia caused significant lipid peroxidation and decrease in the glutathione content of the liver and brain mitochondria (Figs. 6 and 7). Taurine is a well-known biomembrane stabilizer and effectively mitigates oxidative stress and lipid peroxidation [13, 43]. This amino acid is also reported to preserve cellular glutathione reservoirs and boosts antioxidant defense mechanisms [44]. Hence, the antioxidant capacity of taurine could also be involved in its protective properties against ammonia-induced mitochondrial dysfunction. Interestingly, it has been found that the antioxidant properties of taurine might be mediated through the effect of this amino acid on mitochondria [19, 45].

High blood ammonia concentrations also affect hepatocytes and liver function [6]. Therefore, hyperammonemia might act as both: a cause and/or an effect of the hepatic injury. On the other hand, the beneficial effects of taurine on liver function and xenobiotics-induced liver injury have been extensively mentioned in previous investigations [17, 46-48]. Hence, taurine administration could be beneficial in HE and hyperammonemia by correcting liver function and preserving the liver capability for ammonia detoxification.

Ca^{2+} is a well-known inducer of mPT [49]. An ammonia-induced rise in Ca^{2+} levels has been found in cultured cells when exposed to this toxin [50]. Calcium chelators effectively mitigated ammonia-induced mitochondrial injury and cell swelling [50]. On the other side, taurine is a well-known agent, which potentiates the Ca^{2+} sequestering capability of mitochondria [27, 51]. Therefore, this unique property of this amino acid might be beneficial in mitigating ammonia-induced cytotoxicity.

Brain mitochondrial dysfunction in hepatic encephalopathy leads to disturbances in the cerebral energy metabolism [7, 8]. Ammonia affects several pathways, including the tricarboxylic acid cycle (TCA), lactate-malate shuttle, α-ketoglutarate dehydrogenase (α-KGDH), and mitochondrial respiratory chain complexes. Hence, the rate of energy production in cellular mitochondria will be compromised [8, 52, 53].

Several investigations revealed that the administration of L-carnitine, acyl-L-carnitine, and creatine (mentioned as 'energy enhancing' compounds) improved the neurological functions in HE [54, 55]. It has also been reported that L-carnitine mitigated ammonia-induced CNS disorders in mice [54, 56]. Acetyl-L-carnitine has also been shown to improve cerebral energy metabolism by activating mitochondrial enzymes of the electron transport chain [56]. Treatment combined with L-carnitine and acyl-L-carnitine also improved the cognitive functions and reduced behavioral abnormalities in patients with HE [56]. All these findings indicate the role of mitochondria and bioenergetic disturbances in the ammonia-induced CNS complications. The administration of other agents such as L-Ornithine-L-Aspartate also has been shown to affect blood and cerebrospinal fluid ammonia level [57]. Interestingly L-Ornithine-L-Aspartate elevated some amino acids in the CSF fluid, including taurine [57].

mPT plays a crucial role in the bioenergetics failure associated with HE and hyperammonemia. As cell volume regulation is an energy-dependent process, it is likely that mitochondrial dysfunction represents a critical factor in the development of brain edema in HE. Several studies indicate that taurine is localized in mitochondria [58-60]. Taurine also has been demonstrated to be a constituent of modified uridine residues in mitochondrial tRNA [61, 62]. All these findings might indicate the importance of taurine in mitochondrial function, as well as its potential therapeutic applications in hyperammonemia.

It has been reported that taurine preserves mPT in cells [63]. Mitochondrial pH gradient and membrane potential are important factors for mitochondrial function [14, 29, 30]. Hence, chemicals that are able to localize in the mitochondrial matrix and regulate matrix pH are capable of preserving mitochondrial membrane potential. It has been found that taurine is involved in the mitochondrial matrix pH regulation [14, 29, 30]. The buffering property of taurine has also been found to be involved in the preservation of the function of mitochondrial matrix localized enzymes

[14, 29, 30]. Interestingly, acyl-CoA dehydrogenase enzymes are demonstrated to have optimal activity in taurine buffer acyl-CoA dehydrogenase enzymes [14, 29, 64]. Thus, taurine prevents crucial enzyme defects in the hyperammonemic environment.

Brain edema is a major cause of mortality in hyperammonemia [65, 66]. Ammonia-induced brain edema has been directly related to astrocytes swelling [67]. Mechanisms that are involved in astrocyte swelling include oxidative and nitrosative stress, as well as mitochondrial dysfunction [34, 42, 67]. Ammonia has also caused mitochondrial permeability transition in cultured astrocytes [68]. It has been reported that taurine effectively mitigated astrocyte swelling in an *in vitro* system [69]. The effect of taurine on astrocyte swelling could be mediated by its positive effect on cell mitochondria.

Excess glutamic acid (Glu) in the ammonia-intoxicated brain is responsible for the over-activation of NMDA receptors and an excitotoxic response [69, 70]. On the other hand, the anti-excitotoxic effect of taurine also has been shown in several investigations [69, 71, 72]. Taurine might mitigate this response by activating inhibitory GABA receptors [69]. Hence, taurine might also mitigate the NMDA-mediated effects of ammonia in CNS.

In the current investigation, we found that taurine administration preserved the mitochondrial membrane potential and prevented mitochondrial swelling. These findings indicate that taurine is capable of protecting mitochondria as a crucial target of ammonia-induced toxicity in the brain and liver. The effect of taurine on mitochondrial respiratory chain complexes, as well as other enzymes affected by ammonia, requires further research.

We observed that taurine effectively ameliorated the direct effect of ammonia on isolated brain and liver mitochondria. This could make this amino acid a potential candidate against cerebral energy metabolism disturbances in acute and chronic liver injury and hepatic encephalopathy. Indeed, precise and in-depth *in vivo* and clinical evaluations will make clear the effect of taurine on mitochondria and reveal the therapeutic value of this amino acid against hyperammonemia complications.

Acknowledgments

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Disclosure

Authors report no conflict of interest.

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