

Original paper

# Ammonia-induced mitochondrial impairment is intensified by manganese co-exposure: relevance to the management of subclinical hepatic encephalopathy and cirrhosis-associated brain injury

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## Abstract

**Aim of the study:** Hepatic encephalopathy (HE) is a neuropsychiatric syndrome ensuing from liver failure. The liver is the major site of ammonia detoxification in the human body. Hence, acute and chronic liver dysfunction can lead to hyperammonemia. Manganese (Mn) is a trace element incorporated in several physiological processes in the human body. Mn is excreted through bile. It has been found that cirrhosis is associated with hyperammonemia as well as body Mn accumulation. The brain is the primary target organ for both ammonia and Mn toxicity. On the other hand, brain mitochondria impairment is involved in the mechanism of Mn and ammonia neurotoxicity.

**Material and methods:** The current study was designed to evaluate the effect of Mn and ammonia and their combination on mitochondrial indices of functionality in isolated brain mitochondria. Isolated brain mitochondria were exposed to increasing concentrations of ammonia and Mn alone and/or in combination and several mitochondrial indices were assessed.

**Results:** The collapse of mitochondrial membrane potential, increased mitochondrial permeabilization, reactive oxygen species formation, and a significant decrease in mitochondrial dehydrogenase activity and ATP content were evident in Mn-exposed (0.005-1 mM) brain mitochondria. On the other hand, ammonia (0.005-0.5 mM) caused no significant changes in brain mitochondrial function. It was found that co-exposure of the brain mitochondria to Mn and ammonia causes more evident mitochondrial impairment in comparison with Mn and/or ammonia alone.

**Conclusions:** These data indicate additive toxicity of ammonia and Mn in isolated brain mitochondria exposed to these neurotoxins.

**Key words:** brain injury, cirrhosis, energy crisis, hepatic encephalopathy, locomotor dysfunction.

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## Introduction

Hepatic encephalopathy (HE) is a deleterious clinical complication accompanying acute and chronic liver injury [1]. Although the exact cause of HE is not known, there is agreement on the predominant role of

ammonia in the pathogenesis of HE-associated complications [2]. Ammonia is metabolized in the urea cycle by the liver. Damaged livers are unable to metabolize ammonia. Hence, this chemical reaches toxic levels in the systemic circulation. Brain tissue is susceptible to high ammonia concentrations [3, 4]. Ammonia

is a neurotoxin that mostly influences astrocytes in the central nervous system (CNS) [5, 6]. Several direct toxic effects of ammonia on neurons have also been characterized [6]. Ammonia causes brain edema, oxidative stress, and inflammation when its level rises during HE [7]. Consequently, a decline in brain function occurs in patients with HE [7]. Hyperammonemia also affects hepatocytes and liver function [8].

Oxidative stress and its subsequent deleterious events have been recognized as the primary mechanisms for ammonia-induced neurotoxicity [5, 9]. On the other hand, ammonia is a mitochondrial toxin [10-12]. It has been reported that mitochondrial function is impaired and brain energy metabolism is interrupted in chronic and acute models of HE [13-15]. Ammonia negatively affects several vital enzymes that are responsible for energy metabolism in mitochondria [16]. Asymptomatic hepatic encephalopathy due to chronic liver injury, hepatitis, mild cirrhosis, known as “minimal hepatic encephalopathy” or “subclinical hepatic encephalopathy” (SCHE), is a complication which represents as a subclinical elevation in plasma ammonia level [17, 18]. Memory loss, locomotor dysfunction, stupor, and defect in intellectual function are attributed to SCHE [17, 18]. A high rate of SCHE is associated with liver injury and cirrhosis [17, 18]. All these events could affect patients' quality of life.

Manganese (Mn) is an essential trace element incorporated in the structure of several enzymes and metabolic pathways in humans [19]. On the other hand, it has been documented that excess Mn exposure is toxic especially toward the CNS [20-22]. Mn-induced neurotoxicity is clinically revealed as locomotor dysfunction and a Parkinsonism-like syndrome [23, 24]. Oxidative stress and defect in the brain tissue antioxidant systems have been revealed in Mn models of neurotoxicity [21, 25-28]. On the other hand, it has been found that Mn exposure significantly worsened mitochondrial function [25, 29-32].

It has been found that Mn excretion is disturbed in cirrhotic patients [23, 33-36]. As Mn is excreted in the bile, any defect in bile excretion leads to a high body Mn level. A several-fold increase in blood and brain tissue level of Mn has been found in cirrhosis [23, 33-36]. Hence, high brain Mn might play a role in cirrhosis-associated CNS injury.

The current study aimed to evaluate the effect of non-toxic concentrations of ammonia alone or in combination with Mn exposure to evaluate any synergistic mitotoxicity effects of these neurotoxic chemicals. The results might help to improve clinical management of HE-associated CNS complications

especially in chronic cases (e.g., cirrhosis-associated subclinical hyperammonemia).

## Material and methods

### Chemicals

Sodium succinate, fatty acid-free bovine serum albumin (BSA) fraction V, thiobarbituric acid (TBA), 4,2-hydroxyethyl,1-piperazineethanesulfonic acid (HEPES), manganese chloride, dimethyl sulfoxide (DMSO), ammonium chloride, D-mannitol, 3-(N-morpholino)-propane sulfonic acid (MOPS), n-butanol, 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), 2',7'dichlorofluorescein (DCF), sucrose, rhodamine 123 (Rh 123), Coomassie brilliant blue, and ethylene glycol-bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Hydroxymethyl aminomethane hydrochloride (Tris-HCl) and trichloroacetic acid (TCA) were purchased from Merck (Darmstadt, Germany). All salts for preparing buffers were of the analytical grade and obtained from Merck (Darmstadt, Germany).

### Animals

Male C57BL/6 mice (20-25 g) were housed in cages on wood bedding (temperature of  $23 \pm 1^\circ\text{C}$ ; relative humidity of 40%). Animals had free access to tap water and a standard chow diet. Animals received human care and were handled according to the animal handling protocols which were approved by the Institutional Animal Experiment Committee of Shiraz University of Medical Sciences (#95-01-36-12832).

### Brain mitochondria isolation

Brain mitochondria were isolated by the differential centrifugation method as previously described [37, 38]. Briefly, animals were euthanized by cervical dislocation, and their brain was rapidly excised and immersed in ice-cooled ( $4^\circ\text{C}$ ) isolation buffer containing 225 mM mannitol, 1 mM EGTA, 75 mM sucrose, 5 mM HEPES, 0.1% essentially fatty acid-free bovine serum albumin (pH = 7.4) at a 10 : 1 buffer-to-tissue (v : w) ratio [37-39]. The brain was homogenized, and the tissue homogenate was centrifuged (600 g for 10 min at  $4^\circ\text{C}$ ) to remove intact cells and nuclei. The supernatants were further centrifuged (12,000 g, 8 min,  $4^\circ\text{C}$ ) to precipitate the heavy membrane fractions (mitochondria) [38]. The recent step was repeated three times using the fresh mitochondria isolation buffer medium

to increase mitochondrial yield. As mentioned, all manipulations for mitochondria isolation were performed at 4°C or on ice to minimize mitochondrial injury [37, 38].

### Mitochondrial membrane potential

Mitochondrial uptake of rhodamine 123 as a fluorescent probe was used for the estimation of mitochondrial depolarization [37, 40-42]. Briefly, the mitochondrial fractions (1 mg protein/ml) were incubated with 10 µl of rhodamine 123 (final concentration of 10 µM) in a buffer containing 125 mM sucrose, 10 mM HEPES, 65 mM KCl, pH = 7.2 (30 min, 37°C, in the dark). Afterward, samples were centrifuged (15,000 g, 5 min, 4°C) and the fluorescence intensity of the supernatant was monitored using a FLUOstar Omega (BMG Labtech, Germany) multifunctional microplate reader at the excitation and emission wavelength of  $\lambda = 485$  nm and  $\lambda = 525$  nm, respectively [37].

### Mitochondrial swelling assay

Mitochondrial swelling was measured as previously described [37]. Briefly, the isolated mitochondria samples (0.1 mg protein/ml) were suspended in a buffer containing 125 mM sucrose, 10 mM HEPES, 65 mM KCl, and pH = 7.2. The light absorbance (at  $\lambda = 540$  nm) was monitored (30 min, 1-minute intervals) using an Epoch plate reader (BioTek Instruments, Highland Park, USA) with constant shaking and temperature control (30°C) [37, 43]. It is accepted that decreased light absorbance is consistent with an increase in mitochondrial volume. Hence, as mitochondria become more swollen, the differences between the light absorbance of two time points ( $\Delta OD_{540}$  nm) increase [37, 44].

### Mitochondrial dehydrogenase activity

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was applied as a colorimetric method for determination of mitochondrial dehydrogenase activity in isolated mouse brain mitochondria [44-46]. Briefly, mitochondrial suspension (1 mg protein/ml) in a buffer containing 220 mM mannitol, 70 mM sucrose, 2 mM HEPES, and 0.5 mM EGTA, at pH 7.4, was incubated with 0.4% of MTT at 37°C for 30 min in the dark. Samples were centrifuged (12,000 g, 15 min) and the pellet of purple formazan crystals was dissolved in 1 ml dimethyl sulfoxide (DMSO). Afterward, 100 µl of dissolved formazan was added to a 96-well plate, and the optical density

was measured with an Epoch plate reader ( $\lambda = 570$  nm, BioTek Instruments, Highland Park, USA) [44]. For standardization of all data obtained in the current study, sample protein concentrations were determined by the Bradford method [47].

### Estimation of reactive oxygen species (ROS) in isolated brain mitochondria

Mitochondrial ROS measurement was performed using the fluorescent probe DCFH-DA [37]. Briefly, isolated brain mitochondria were incubated in a respiratory buffer containing 65 mM KCl, 125 mM sucrose, 10 mM HEPES, 5 mM sodium succinate, and 20 µM  $Ca^{2+}$ , at pH 7.2 [37]. DCFH-DA was added (final concentration, 10 µM) to mitochondria and then incubated for 30 min at 37°C in the dark. Then, the fluorescence intensity of DCF was measured using a FLUOstar Omega (BMG Labtech, Germany) multifunctional fluorescent microplate reader ( $\lambda_{excitation} = 485$  nm and  $\lambda_{emission} = 525$  nm) [37, 44].

### Mitochondrial ATP level

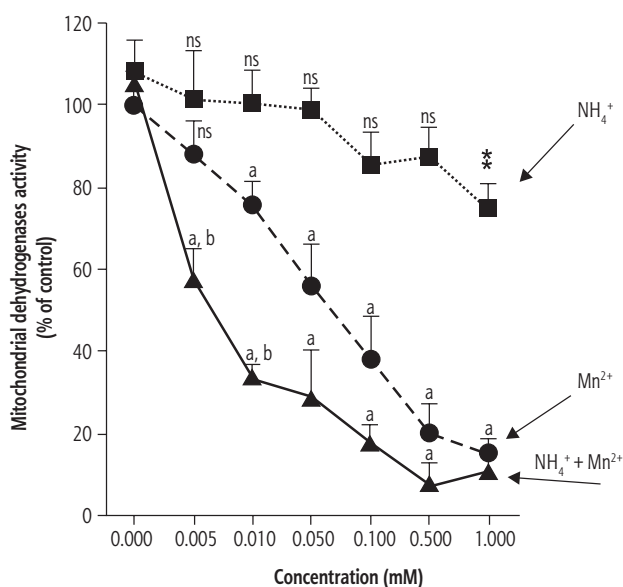
A luciferase-luciferin-based kit (ENLITEN from Promega, USA) was used to assess mitochondrial ATP content [48-50]. Samples and buffer solutions were prepared based on the kit instructions. Isolated brain mitochondria (1 mg/ml protein) were treated with 100 µl of trichloroacetic acid (0.3% w : v) and centrifuged (15,000 g, 20 min, 4°C). Afterward, 100 µl of the supernatant was added to 100 µl of the ATP kit and the luminescence intensity of samples was measured ( $\lambda = 560$  nm) using a FLUOstar Omega (BMG Labtech, Germany) multifunctional microplate reader [48-50].

### Statistical analysis

Data are given as the mean  $\pm$ SD. Data comparison was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test as the *post hoc* test. Differences were considered statistically significant when  $p < 0.05$ .

### Results

There were no significant changes in mitochondrial dehydrogenase activity in isolated brain mitochondria exposed to ammonia up to 500 µM. On the other hand, concentrations of ammonia higher than 1 mM significantly decreased mitochondrial dehydrogenase activity in comparison with the control group. Mn administration caused a significant decrease in



Data are presented as mean  $\pm$ SD (n = 8). <sup>a</sup>Indicates significantly different as compared with control (0 mM) ( $p < 0.01$ ). <sup>b</sup>Indicates significantly different as compared with the same concentration of  $NH_4^+$  and/or  $Mn^{2+}$ -treated mitochondria ( $p < 0.05$ ). ns – not significant as compared with control (0 mM).

**Fig. 1.** Isolated brain mitochondrial dehydrogenases activity in the presence of manganese ( $Mn^{2+}$ ) and ammonia ( $NH_4^+$ )

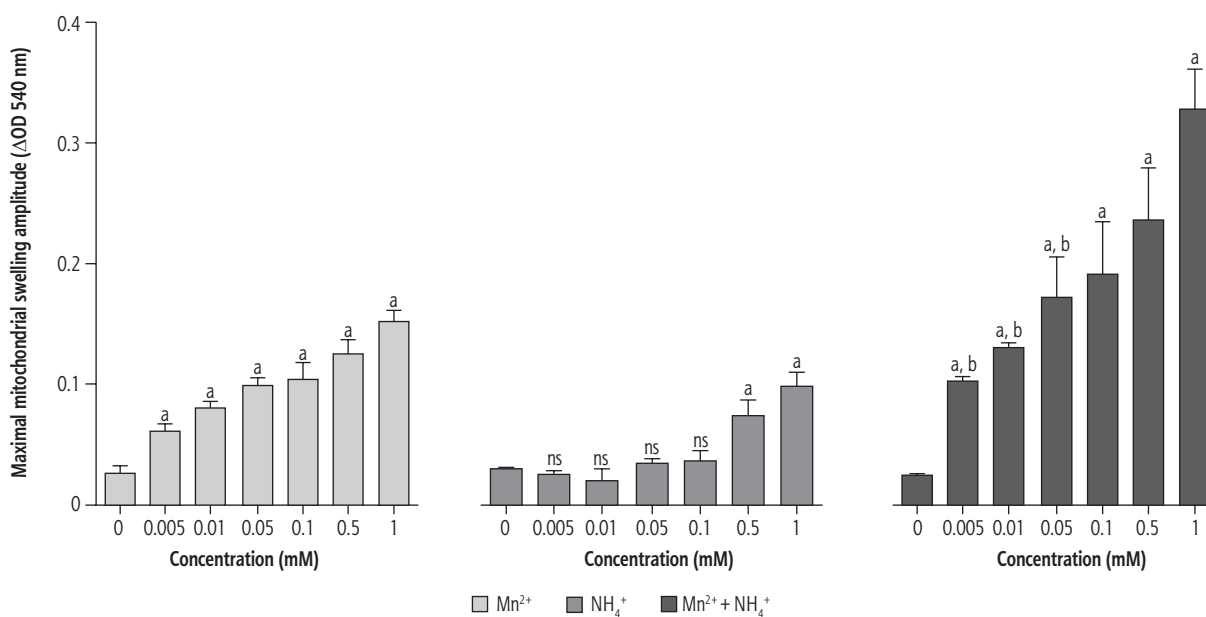
mitochondrial dehydrogenase activity even at low concentrations of this trace element. On the other hand, it was found that the effect of combined Mn and ammonia on brain mitochondrial dehydrogenase activity was significantly higher than the sole administration of these chemicals (Fig. 1).

Further evaluation of mitochondrial function revealed significant mitochondrial permeabilization and swelling in the Mn-treated groups. Significant mitochondrial swelling was also identified in 1 mM ammonia-exposed isolated brain mitochondria. On the other hand, it was found that co-exposure of Mn and ammonia caused significantly higher mitochondrial swelling amplitude in comparison with the Mn and/or ammonia-treated group (Fig. 2).

A significant collapse of mitochondrial membrane potential was evident in Mn-exposed mitochondria in comparison with the control group. Mitochondria depolarization was also detected in ammonia 1 mM group. It was found that Mn and ammonia co-administration exacerbated ammonia-induced mitochondrial depolarization (Fig. 3).

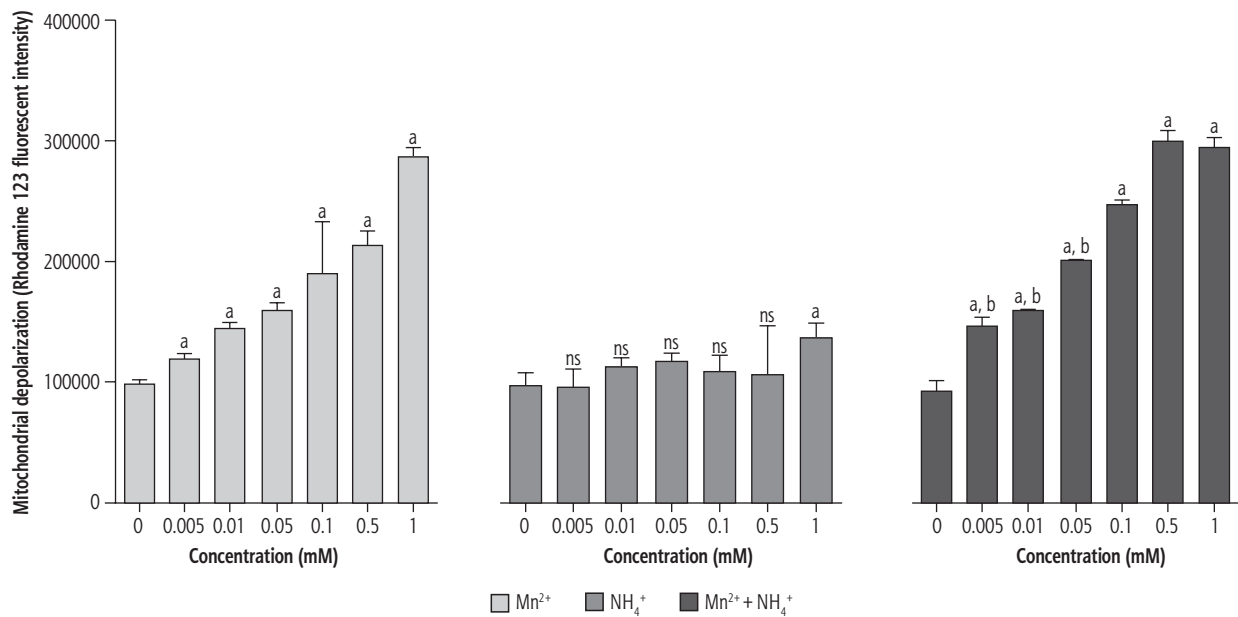
Mitochondria-mediated ROS formation was hastened in Mn-treated brain mitochondria as compared with the control group. Significant ROS also was detected in the ammonia (1 mM)-treated group. On the other hand, more ROS were formed when isolated mice brain mitochondria were co-exposed to Mn and ammonia in comparison to the sole Mn and ammonia group (Fig. 4).

Mitochondria ATP content was significantly decreased when mice brain mitochondria were exposed to Mn (0.005-1 mM). Ammonia (1 mM) treatment also caused a significant decrease of mitochondrial ATP content in comparison with the control group. It was found that co-exposure to Mn and ammonia caused significant depletion of mitochondrial ATP content in



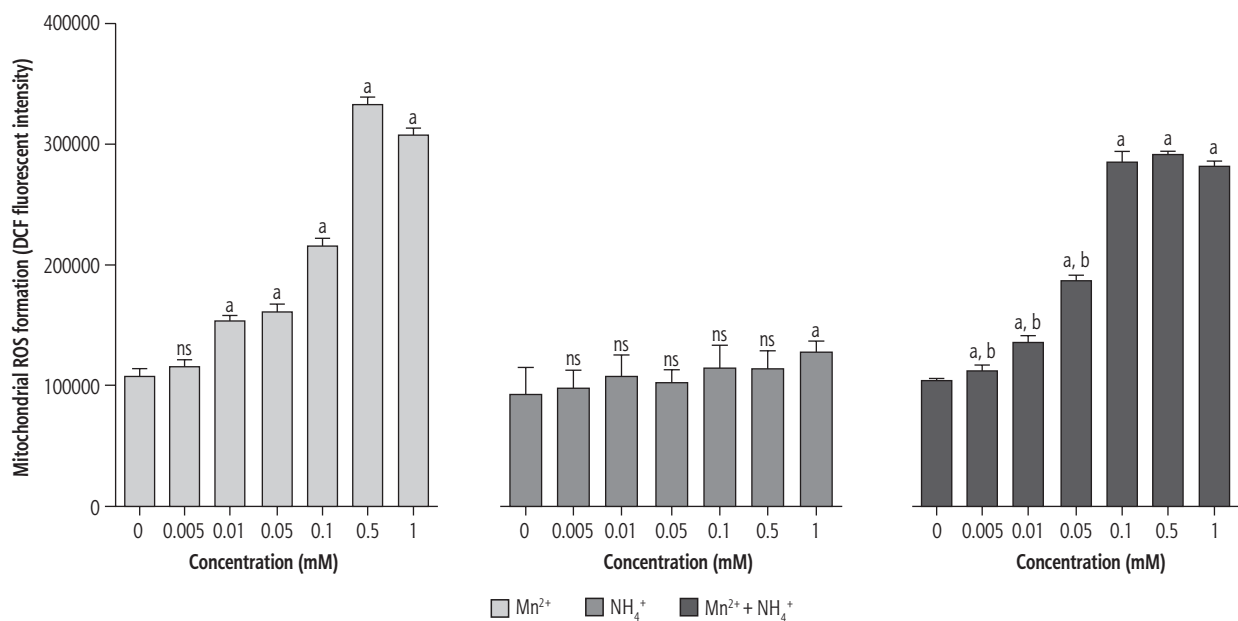
Data are given as mean  $\pm$ SD (n = 8). <sup>a</sup>Indicates significantly different as compared with control (0 mM) ( $p < 0.01$ ). <sup>b</sup>Indicates significantly different as compared with the same concentration of  $NH_4^+$  and/or  $Mn^{2+}$ -treated mitochondria ( $p < 0.05$ ). ns – not significant as compared with control (0 mM).

**Fig. 2.** Ammonia ( $NH_4^+$ ) and manganese ( $Mn^{2+}$ )-induced mitochondrial swelling and permeabilization



Data are shown as mean  $\pm$ SD ( $n = 8$ ). <sup>a</sup>Indicates significantly different as compared with control (0 mM) ( $p < 0.01$ ). <sup>b</sup>Indicates significantly different as compared with the same concentration of NH<sub>4</sub><sup>+</sup> and/or Mn<sup>2+</sup>-treated mitochondria ( $p < 0.05$ ). ns – not significant as compared with control (0 mM).

**Fig. 3.** Mitochondrial depolarization in the presence of ammonia (NH<sub>4</sub><sup>+</sup>) and manganese (Mn<sup>2+</sup>)



Data are shown as mean  $\pm$ SD ( $n = 8$ ). <sup>a</sup>Indicates significantly different as compared with control (0 mM) ( $p < 0.01$ ). <sup>b</sup>Indicates significantly different as compared with the same concentration of NH<sub>4</sub><sup>+</sup> and/or Mn<sup>2+</sup>-treated mitochondria ( $p < 0.05$ ). ns – not significant as compared with control (0 mM).

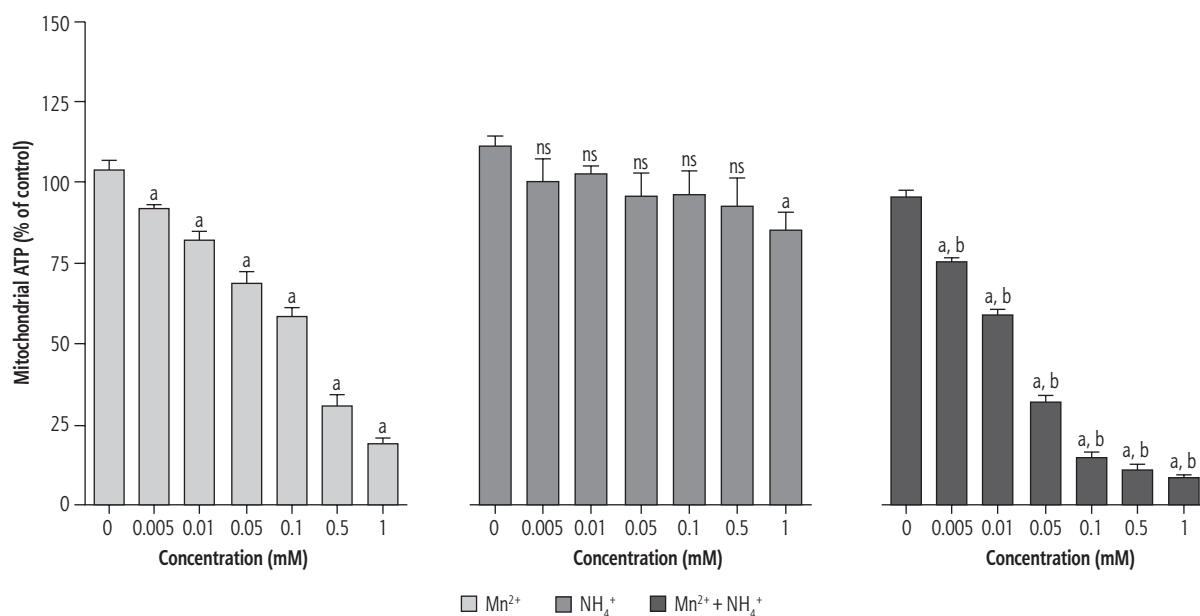
**Fig. 4.** Increased mitochondrial reactive oxygen species level in the presence of manganese (Mn<sup>2+</sup>) and ammonia (NH<sub>4</sub><sup>+</sup>)

comparison with Mn and/or ammonia-treated groups (Fig. 5).

## Discussion

Chronic liver injury and cirrhosis are the typical outcomes of a variety of liver diseases and toxic insults.

Cirrhosis is a chronic liver injury with multifaceted clinical features. It has been well documented that cirrhosis is associated with chronic hepatic encephalopathy (HE). Cirrhosis-associated HE is characterized as disturbances in locomotor function, fatigue, the decline in intellectual capacity, sleep disturbances, and altered patient mood [51-54]. All these changes affect patients' quality of life.



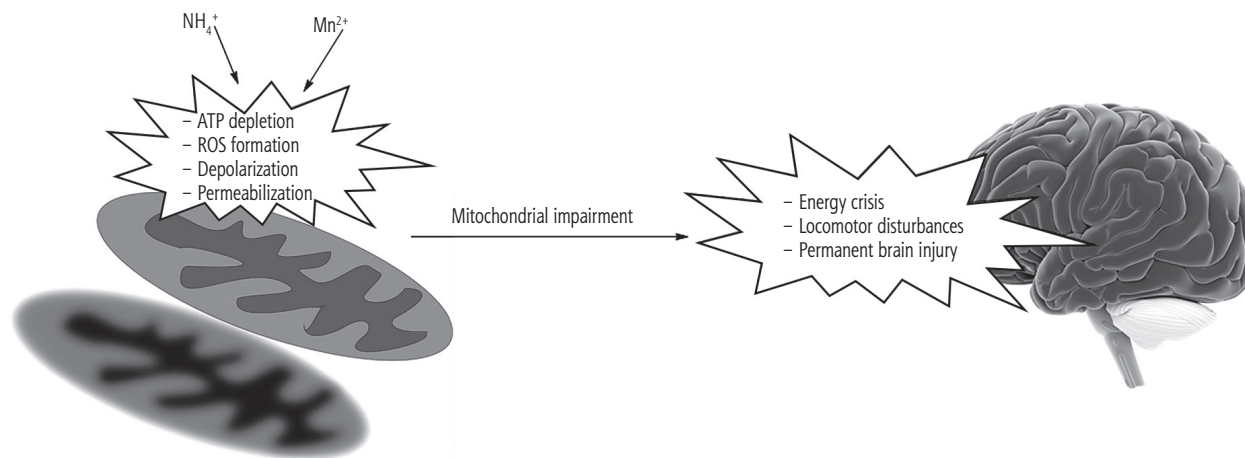
Data are shown as mean ±SD (n = 8). <sup>a</sup>Indicates significantly different as compared with control (0 mM) (p < 0.01). <sup>b</sup>Indicates significantly different as compared with the same concentration of NH<sub>4</sub><sup>+</sup> and/or Mn<sup>2+</sup>-treated mitochondria (p < 0.05). ns – not significant as compared with control (0 mM).

**Fig. 5.** Ammonia (NH<sub>4</sub><sup>+</sup>) and manganese (Mn<sup>2+</sup>)-induced decrease in mitochondrial ATP content

Impaired liver capacity of ammonia detoxification and blood and brain ammonia levels have been strongly implicated in the pathogenesis of cirrhosis-associated HE [4, 6, 54]. Several cellular and molecular mechanisms have been identified for ammonia toxicity. Oxidative stress and its subsequent events play a major role in the mechanism of ammonia neurotoxicity [5, 55, 56]. At the cellular level, mitochondrial impairment and cellular energy (ATP) crisis are involved in the mechanism of ammonia toxicity [8, 11, 12, 56-58]. Impaired mitochondria energy metabolism, significant mitochondrial permeabilization, enhanced mito-

chondria-mediated ROS formation, and release of cell death mediators from cellular mitochondria are connected with the effects of ammonia on the brain tissue [8, 11, 12, 56-58] (Fig. 6).

Manganese is a trace element physiologically incorporated in the structure of several vital enzymes in the human body [19]. On the other hand, it has been found that excess Mn exposure is associated with several adverse effects, especially in the brain [20-22]. Physiologically, Mn is excreted in the bile [23, 33-36]. Hence, any defect in the bile flow could lead to Mn accumulation in the body [23, 33-36]. It has been found



**Fig. 6.** Schematic representation of the additive effects of manganese and ammonia on brain mitochondrial function. Impaired brain mitochondrial function could play a critical role in CNS injury during cirrhosis and chronic hepatic encephalopathy. Brain energy crisis, disturbances in locomotor activity, coma, and permanent brain injury might ensue from hepatic encephalopathy

that cirrhosis is associated with an elevated body Mn level [23, 33-36]. Up to a 7-fold increase in the blood and brain Mn level is documented in cirrhotic patients [33, 59]. Mn is accumulated in the brain tissue, where it manifests its toxic properties. A wide range of impairment including locomotor dysfunction and mood abnormalities has been reported in association with brain Mn deposition [21, 24, 51]. It has been found that cirrhosis-associated locomotor dysfunction might be associated, at least in part, with high brain Mn level [18, 22, 33, 34].

Cellular mitochondria are among the major targets of Mn toxicity [25, 29-32]. Mn is accumulated in the mitochondrial matrix through  $\text{Ca}^{2+}$  transporters [60, 61]. Severe collapse of the mitochondrial membrane potential and inhibition of mitochondria respiratory chain complexes are reported in association with mitochondrial Mn exposure [25, 29-32, 60, 61].

In chronic liver injury (e.g., cirrhosis) both Mn and ammonia are present in the brain tissue. As mentioned, cellular mitochondria are affected by ammonia and Mn. In the current study, it was found that lower levels of ammonia become more toxic in the presence of Mn. A more significant decrease in brain mitochondria dehydrogenase activity, the collapse of mitochondrial membrane potential, mitochondrial permeabilization, and depletion of mitochondrial ATP content were detected when brain mitochondria were co-exposed to both Mn and ammonia (Fig. 6). On the other hand, Mn adversely affected mitochondrial function even at low concentrations (0.01 mM). These data suggest the importance of brain Mn accumulation in cirrhosis-associated CNS complications. This might indicate the importance of effective therapeutic options which consider strategies against both Mn and ammonia (Fig. 6). As mitochondria are among the major cellular targets affected by ammonia and Mn, regulating mitochondrial function and preserving cellular energy (ATP) status at a higher level might affect Mn and ammonia neurotoxicity in cirrhotic patients.

Some studies have mentioned the importance of Mn chelation therapy as an option against Mn neurotoxicity [62, 63]. However, the effectiveness of such strategies has been questioned. Chelation therapy might disturb the homeostasis of other metals in the human body or lead to complications such as nephrotoxicity. Hence, other ancillary therapeutic options are required to manage central nervous system complications associated with manganese or ammonia. Some safe and clinically applicable chemicals have been identified to protect the brain in situations such as HE [64-68]. Hence, mitigating brain oxidative stress and protecting cellular mitochondria could be a viable

therapeutic approach against ammonia and Mn neurotoxicity (Fig. 6).

The data obtained in the current investigation might provide some clues that ammonia-induced mitochondrial impairment and neurotoxicity are exacerbated in the presence of Mn. This could help to improve clinical management of chronic liver injury (e.g., cirrhosis)-associated CNS complications.

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## Disclosure

Authors report no conflict of interest.

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