Thioacetamide-Induced Liver Cirrhosis Alters Oxidative Stress Balance and Induces Mitochondrial Respiratory Chain Inhibition in the Brain of Cirrhotic Rats.

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Abstract

Hepatic encephalopathy (HE) constitutes an intriguing complication characterized with neuropsychiatric symptoms that occurs as a consequence of acute or chronic liver disease. Subjects with hepatic encephalopathy are associated with elevated blood ammonia and other metabolites which are neurotoxic, ultimately leading to neuronal death due to oxidative stress. Evidences from previous studies suggest the implication of mitochondrial dysfunctions in oxidative stress conditions. In the current study we investigated brain oxidative metabolism and activities of mitochondrial respiratory chain complexes in rat model of Thioacetamide (TAA) –induced chronic cirrhosis. The activities of critical enzymes for mitochondrial function, Mn-superoxide dismutase, complex I and complex III were decreased progressively in the mid-brain cortex. We also evaluated creatine kinase (CK) activity as a marker of energy homeostasis that is shown to be inhibited by free radicals, and probably involved in the pathogenesis of hepatic encephalopathy, as well as glutathione peroxidase (GSH-PX), catalase and superoxide dismutase activities. SOD and catalase activities were decreased significantly but on the contrary GSH-PX activity is significantly increased at all studied time points. These results suggest that oxidative stress-associated inhibition of mitochondrial respiratory chain complexes and brain CK activities following hepatic failure may contribute, in part, to the early-stage pathogenesis of hepatic encephalopathy.

Keywords: Liver; Brain; Reactive oxygen species; Mitochondria; Hepatic encephalopathy.

Introduction

The association between liver disease and cerebral dysfunction had already been recognized 2500 years ago when Hippocrates stated that "those who are mad on account of phlegm are quiet, but those on account of bile are vociferous, vicious, and do not keep quiet" [1]. There are scant data on the association between liver disease and brain dysfunction ranging from mild neuropsychiatric and psychomotor dysfunction, impaired memory, increased reaction time, shortened attention span, sleep-wake inversions, brain edema, and poor concentration to severe features such as confusion, stupor, coma, and eventually death [2].

The central nervous system is vulnerable to oxidative stress, especially when a toxicant can modify the physiological balance between pro- and anti-oxidant mechanisms [3]. Mitochondria play essential role in energy metabolism through controlling the generation of reactive oxygen species, and regulation of apoptosis [4]. Moreover, ROS are produced continuously as a by-product of aerobic metabolism. Superoxide can be produced as a result of the one-electron reduction system within the mitochondrial electron transport chain. Experiments using mammalian and bird mitochondria from various tissues have identified two main sites capable of producing ROS within the electron transport chain: complex I and center o of complex III [5].

Glutathione is the brain master's antioxidant and protects neurons from the harmful effects of free radicals. Many neurological and psychiatric disease processes are characterized by abnormalities in glutathione metabolism and antioxidant defenses [6]. Selective reductions in GSH levels, which precede losses in mitochondrial complex I activity, have been reported to occur not only in Parkinson's disease but also in toxin models associated with it [7].

Because energy is necessary to maintain the development and regulation of cerebral functions, it has been postulated that alterations in CK activity may participate in a neurodegenerative pathway leading

to neuronal loss in the brain [8]. Recent findings have reinforced this hypothesis, showing that CK activity is severely reduced in several neurodegenerative diseases [9].

The molecular mechanism involved in hepatic neurotoxicity has yet not been clarified, but it has been suggested that building up of toxic substances in the blood may alter the oxidative homeostasis, energy metabolism and mitochondrial complexes activities systemically. Therefore, in the present study, we investigated in a time course manner the effect of Thioacetamide (TAA) administration on liver functions and associated alterations of the brain mitochondrial complexes I and III activities. In addition to we identified biochemical systems in the brain that are susceptible to ROS toxicity.

Materials and Methods

Animals and experimental design

45 Male Wistar Rats were kept in dams while receiving the drugs until they were sacrificed. The dams had free access to water and to a standard commercial chow containing 20.5% protein (predominantly soybean supplemented with methionone), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash, and 10% moisture. Temperature was maintained at 22 ±2°C, with a 12–12-h light dark cycle. The Principles of Laboratory Animal Care were followed in all the experimental protocols and was approved by the Ethics Committee for Animal Research at Faculty of Medicine, Alexandria University.

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Rats were divided into two major groups: The first served as controls and received a subcutaneous injection of physiological saline while the second group was induced by intraperitoneal (i.p.) administration of Thioacetamide (TAA) (200 mg/kg twice weekly for 18 weeks). Six to eight rats from each group were killed by cervical dislocation at the time intervals 6, 12 and 18 week(s) post lesion-induction.

Liver cirrhosis progression was followed up by measuring hepatic function indices. Brains were removed and immediately frozen in liquid nitrogen then stored in -80°C. Sera were collected for biochemical analyses. Mid-brain cortex of dissected tissues was used for biochemical analyses. The protein content in brain homogenates was determined by the method of Lowry et al [10] using serum bovine albumin as the standard.

Sera biochemical analyses

Aspartate transaminase (AST) **activity** was determined according to Reitman and Frankel [11] previously described method. Briefly, sera samples were incubated with buffer/ substrate reagent mixture (phosphate buffer 100 mM, pH7.2 containing 80 mM L-aspartae, and 4.0 mM α-ketoglutarate) for exactly 30 min at 37°C. Then the color reagent (4.0mM 2, 4-Dintrophenylhydrazine) was added, and tubes were allowed to stand for exactly 20 min at RT before adding 0.4 M NaOH. Samples were incubated for 5min at RT, then the absorbance of were monitored against reagent blank at 546nm within 1h. Sera AST activities were calculated and represented as U/L.

Alanine transaminase (AlT) **activity** was determined according to Reitman and Frankel [11] previously described method. Briefly, sera samples were incubated with buffer/ substrate reagent mixture (phosphate buffer 100 mM, pH 7.2 containing 80 mM DL-alanine, and 4.0 mM α-ketoglutarate) for exactly 30 min at 37°C. Then the color reagent (4.0 mM 2, 4-Dintrophenylhydrazine) was added, and tubes were allowed to stand for exactly 20 min at RT before adding 0.4 M NaOH. Samples were incubated for 5min at RT, then the absorbance of were monitored against reagent blank at 546nm within 1h. Sera ALT activities were calculated and represented as U/L.

Determination of albumin level was carried out according to Doumas et al, [12] method in which a buffered solution of bromocresol green forms a green colored complex with albumin, where; the intensity of color is directly proportional to the amount of albumin present in sample. Briefly, color reagent (0.18 g/d bromocresol green in succinate buffer, 74 mM, pH 4.2) is added to standard (4.0 g/dl bovine serum albumin) or plasma samples and incubated at RT for 5 min. developed color is measured at 623 nm within 30 min. Albumin concentration of each sample was calculated as g/dl in reference to standard.

Determination of total proteins level was performed using the colorimetric method described by Henry [13]. In brief, sera proteins form violet color complex when react with cupric ions in an alkaline solution (18mM potassium sodium tartrate, 12 mM potassium iodide, 0.2 mM sodium hydroxide, 6.0 mM copper sulfate). The intensity of developed color directly proportional to the amount of proteins presents when compared to standard solution and calculated as g/dl.

Assay of oxidative stress markers

Superoxide dismutase activity was estimated according to Misra and Fridovich [14] method based on its ability to inhibit the autoxidation of epinephrine in an alkaline medium (pH 10.2).

Catalase Activity was determined based on the disappearance of H_2O_2 by antioxidant enzymes in the tissue homogenate at 25°C [15].

The absorbance of chromogen was measured spectrophotometrically at 420 nm. Catalase activities were calculated as unit/g protein/min.

GSH-PX Activity dissected mid-brain cortex was washed with ice-cooled buffer (140 mM-KCl/5mM-potassium phosphate solution). Glutathione peroxidase activity in tissue homogenates was determined by following up the absorbance rate of NADPH oxidation coupled to glutathione peroxidase activity. The activity was expressed as nmol of NADPH oxidized/min per mg of protein [16].

GSH level: Estimation of GSH was performed according to the method of Moron et al [17]. In this method 5, 5-dithiobis 2-nitrobenzoic acid (DTNB) is reduced by -SH groups to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The nitro mercaptobenzoic acid anion released has intense yellow color and can be used to measure -SH groups at 412 nm.

Lipid peroxidation: Lipid peroxidation contents in mid-brain cortex sections was performed according to Cini et al, [18] method using microsomes crude homogenates. Lipid peroxidation was monitored by the formation of thiobarbituric acid reactive substances (TBARS). Briefly, after extraction with n-butanol and pyridine (15:1 v/v), the amount of TBARS formed was determined by measuring the absorbance of the organic layer at 532 nm. In parallel TMP was used as an external standard. Results are reported as μmol malondialdehyde (MDA)/ g protein.

Creatine Kinase Activity

Creatine kinase activity was assayed as described before [19]. The reagents concentrations and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls were carried out to measure chemical hydrolysis of phosphocreatine. The color was developed by the addition of 0.1-ml 2% ®-naphtol and 0.1ml 0.05% diacetyl in a final volume of 1 ml and read after 20 min at 540 nm. CK activity expressed as U/mg protein.

Preparation of Mitochondrial Fractions and measurements of Mitochondria Complexes Activities

Dissected mid brain tissues were homogenized for mitochondria extraction using sucrose density gradients method as described before [20]. Submitochondrial particles were prepared as demonstrated [21]. Sub-mitochondrial particles prepared from the mitochondria isolated brain tissues (10 μg) were used for the assay of the different mitochondrial electron transport chain complexes activities.

Assay of NADH dehydrogenase (Complex I) activity was performed at 340nm using acceptor 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (80 μM) and 200 μM NADH as donor, in 10 mM Tris (pH 8.0) buffer containing 1mg/ml BSA, 0.24 mM KCN and 0.4 μM antimycin A (Complex III inhibitor) for 5 min to permeabilize the mitochondrial internal membrane to NADH, sample was incubated in distilled H2O for 3 min at 37°C. The addition of 5 μM rotenone allowed us to quantify the rotenone-sensitive activity [22].

Assay of Complex III activity was performed at 550 nm using 40 μM oxidized cytochrome c as the acceptor and 80 μM decylubiquinol as the donor in a medium containing 10 mM KH2PO4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP for 2 min. The addition of 1 μM antimycin A allowed us to distinguish between the reduction of cytochrome c catalyzed by the complex III and the non-enzymatic reduction of cytochrome c by the reduced quinine [23].

Statistical Analysis

All experiments were performed in duplicate or triplicate independently and typical graphs are presented in some cases data are expressed as Mean ± SD. Data were analyzed by Student's t test and difference was considered significant from control (zero time) when $p < 0.01$. One way ANOVA test was used to compare the statistical difference between groups, the results were considered significant when $p < 0.05$.

Results

Thioacetamide (TAA) is an established technique to produce liver failure model of HE. The increased levels of ALT and AST are used as markers to ascertain different grades of liver failure. As compared to the control rats, ~2 to 5 fold increase in ALT and ~ 3 to 6 fold increase in AST were detected in the sera of the TAA induced group (Figure 1).

The general decline in liver functions and cirrhosis development were also confirmed by measuring sera levels of albumin and total proteins. Albumin and total protein levels, were reduced significantly ($p < 0.05$) post TAA-induction (Figure 1). We also confirmed the phase progression of cirrhosis development by histological sections staining using hematoxylin and eosin (data not shown).

Generation of reactive oxygen species (free radicals) and oxidative stress imbalance are an important inducer of neuronal cell death. Therefore lipid peroxidation was assessed in the brain homogenates of TAA-treated rats by measuring MDA level (a stable product of lipid peroxidation). According to Table 1 & Figure 2, as compared to the control rats at all-time points post-lesion induction the acute liver failure rats showed significant increase in the cerebral cortex MDA level. Moreover, levels of glutathione (GSH) were reduced significantly ($p < 0.05$) during the eighteen weeks of follow up period compared to the controls at weeks 12 and 18. On the contrary, activities of glutathione peroxidase (GPX) were significantly increased ($p < 0.05$) in the TAA-induced group during the follow up period (Table 1 & Figure 2).
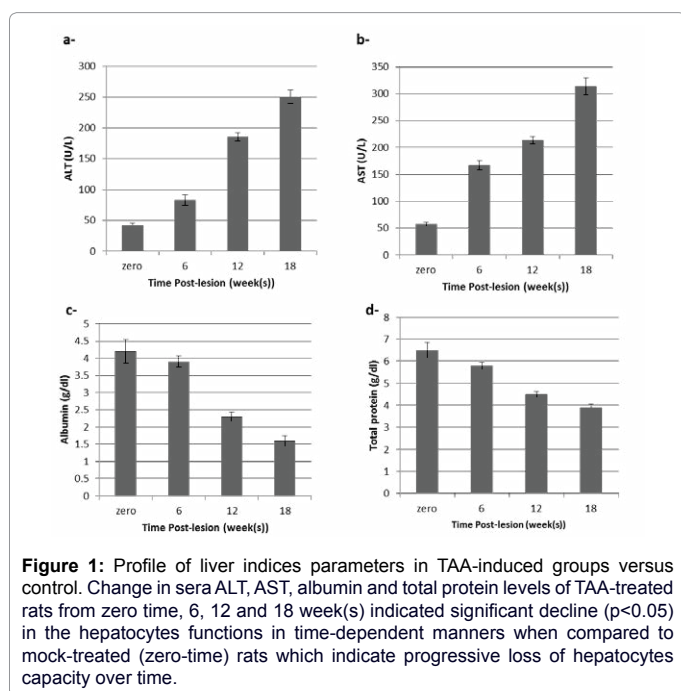
Experimental group (TAA-induction time in weeks)	GPX (U/g.protein)	GSH (mg/g. protein)	MDA (μmol/g. protein)	Catalase (U/g. protein)	SOD (U/g.protein)
			Mean ± SD*		
0	29 ± 2.1234	40 ± 2.134	25.45 ± 1.654	2.33 ± 0.143	4.33 ± 0.145
6	37 ± 2.243	47 ± 3.652	41.34 ± 3.42	2.14 ± 0.223	3.14 ± 0.213
12	64 ± 4.4653	31 ± 1.87	53.5 ± 3.764	1.83 ± 0.102	2.83 ± 0.20654
18	92 ± 5.764	10 ± 2.983	45.7 ± 4.32	1.53 ± 0.1763	2.13 ± 0.1943

*Significance from zero-time at $p < 0.05$

Table 1 Oxidative stress markers in mid-brain tissues of induced rats during 18 weeks of TAA-induction.

In addition to, the antioxidant enzymes catalase and superoxide dismutase (SOD) in the brain tissues of rats exposed to the TAA (dose of 200 mg/kg) for 18 week, registered a highly significant decrease ($p < 0.05$) in their activities compared to controls (Table 1 & Figure 2) at weeks 12 and 18.

Because CK plays an essential role in brain energy metabolism, we followed up its activity during the eighteen weeks of induction. Our results indicated a slight elevation at week 6 of TAA-administration followed by significant reduction ($p < 0.05$) afterwards associated with liver cirrhosis development (Figure 3). The initial elevation of CK activity may occur in correlation with early events of mitochondrial function declination as a compensatory mechanism for ATP production.

To test the possible involvement of TAA-induced liver cirrhosis in altering brain mitochondrial respiratory complexes I and III, we measured their activities in sub-mitochondrial particles (20 μg proteins determined in a preliminary study) prepared from cortex brain homogenates. Results are expressed as fold change in the activity of treated rats from control. Complexes I and III activities were reduced significantly ($p < 0.05$) in a time-dependent fashion following TAA-induced hepatic lesion compared to the control groups (Figure 4). The reduction of complexes I and III activities over time reflects the possible implication of mitochondrial dysfunction as early event during hepatic encephalopathy (Figure 5).

Discussion

Reactive oxygen species have been implicated in the pathophysiology of acute and chronic liver disease. Murphy et al [24] demonstrated the presence of oxidative stress in brain tissue in the presence of liver failure. Mitochondrial dysfunction and morphological damage are present, and mitochondrial permeability transition (MPT) can be formed with the consequent extrusion of its matrix content [25]. Oxidative stress is responsible, then, for the MPT in mitochondria, and lipids and proteins peroxidation in its membranes. Oxidative stress depletes ATP by inhibiting glycogenesis, mitochondrial functions… etc, thus induces energy deficiency.

Because increased oxidative stress could be one of the mediating factors in the pathogenesis of hepatic encephalopathy toxicity in the brain, in the present study an attempt was made to collect comprehensive information both on mitochondrial complexes I and III activities, lipid peroxidation and activities of enzymatic antioxidants together for the conditions of hepatic encephalopathy associated with TAA-induced liver cirrhosis in rat in a time-course fashion.

It is known that pathological states and mitochondrial dysfunction often lead to the excessive generation of free radicals and subsequent
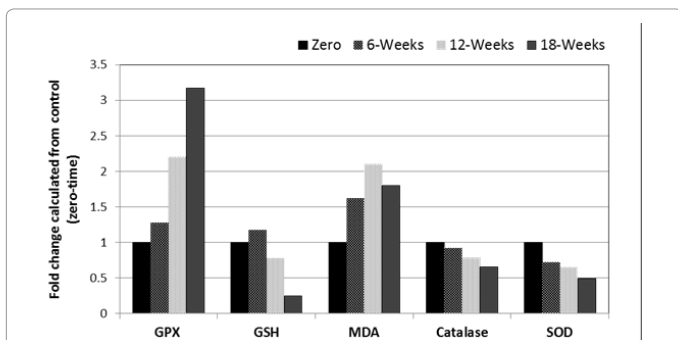

Figure 1: Profile of liver indices parameters in TAA-induced groups versus control. Change in sera ALT, AST, albumin and total protein levels of TAA-treated rats from zero time, 6, 12 and 18 week(s) indicated significant decline ($p < 0.05$) in the hepatocytes functions in time-dependent manners when compared to mock-treated (zero-time) rats which indicate progressive loss of hepatocytes capacity over time.

Figure 2: Antioxidant markers profile of TAA-induced rats. Oxidative stress-related markers including GSH and Lipid peroxidation product Malondialdehyde (MDA) as well as GPX, catalase and SOD activities were determined in brain homogenates of TAA-treated rats. Initially at week-6 of induction, the results indicate significant elevation in GPX and GSH at week 6 as a compensatory mechanism for protection against systemic toxicity. MDA level is shown to be significantly elevated during the 18th weeks of chronic induction. In addition to, activities of SOD and catalase in brain homogenate of cirrhotic rats reduced significantly (p<0.05) compared to normal rats.
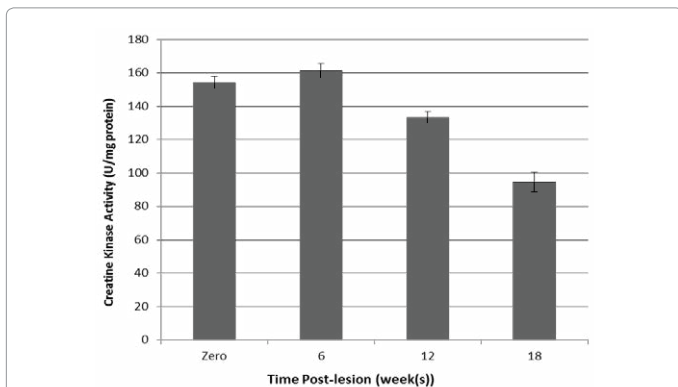

Figure 3: Effect of administration of TAA on creatine kinase activity in rat brain homogenate. The results indicated significant reduction in CK activity (p<0.05) compared to control rats staring form week 12 for 6 weeks which indicate.
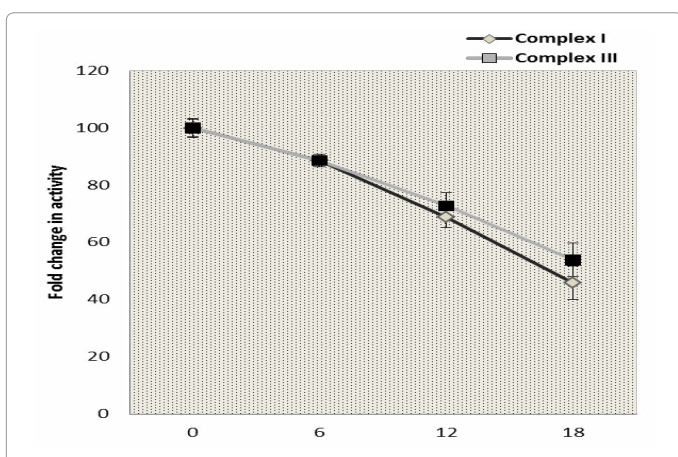

Figure 4: Effect of TAA-induced liver cirrhosis in rats on the mitochondrial complex I and complex III activities. Brain tissues are vulnerable to oxidative stress generated by exposure to toxic agents. Mitochondrial contributes significantly to intracellular reactive oxygen species production in the presence of neurotoxic substances. The results indicate significant reduction (p<0.05) in Complexes I and III activities from weeks 6 to 18 of TAA systemic administration, implying possible role of mitochondrial dysfunction during hepatic encephalopathy pathogenesis.
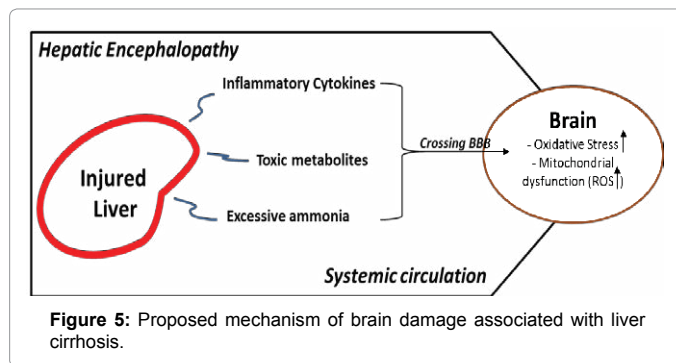

Figure 5: Proposed mechanism of brain damage associated with liver cirrhosis.

oxidative damage [26]. Mitochondrial dysfunction and its relation to neuroinflammation and neurodegeneration have been reported in many studies.

The mitochondrial respiratory chain consists of five complexes, namely complexes I, II, III, IV, V], which catalyze the phosphorylation of adenosine diphosphate to adenosine triphosphate, working as an integrated system composed of a total of five protein complexes [27]. Under normal physiological conditions, 1–5% of the oxygen is converted to ROS [28]. Mitochondria are major sources of reactive oxygen species (ROS). The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron-transport chain, namely at complex I (nicotinamide adenine dinucleotide dehydrogenase) and complex III (ubiquinone-cytochrome c reductase) [29]. Under normal metabolic conditions, complex III is the main site of ROS production [30].

As a consequence of altered electron transport to neuronal viability if severe enough, electron transport inhibition compromises the ability to generate an electrochemical gradient and, therefore, decreases the rate of ATP synthesis [31]. Our results indicate that TAA-induced liver cirrhosis is accompanied with gradual loss of rat brain mitochondrial complexes I and III activities. This is in agreement with Chadipiralla et al., [32]. The inhibition of the respiratory complexes III and I might lead to the increased production of free radical resulting in oxidative stress and cerebral energy disturbances thereby leading to mitochondrial swelling and further contributing to the pathogenesis of hepatic failure.

Selective reductions in GSH levels, which precede losses in mitochondrial complex I activity, have been reported to occur not only in Parkinson's disease but also in toxin models associated with it [33]. Complex I is known to possess active thiol groups and is extremely sensitive to the thiol status in cells. Depletion of reduced glutathione (GSH) may lead to inhibition of complex I activity which is consistent with our data that show a significant reduction of GSH level at weeks 12 and 18 of TAA-treatment.

Generation of ATP, hence creatine kinase (CK) activity, is critical for central nervous system function. CK knock-out mice display a significant neurological phenotype [34]. In the present study brain CK activity was reduced during induction of liver cirrhosis. We hypothesize that increased lipid peroxidation and generation of ROS through reduction in mitochondrial complexes I and III activities may contribute to the reduced activity of brain CK.

It is known that in mammals, superoxide dismutase and peroxide-metabolizing enzymes, including but not limited to, catalase and glutathione peroxidase represent an efficient defense system against hazards of lipid peroxidation. The data presented here also indicate an alteration in the levels of brain antioxidants components in response to TAA-induced liver cirrhosis in rats.

Levels of reduced glutathione decreased while GSH-PX activity increased significantly during cirrhosis induction. An elevation of GSH level was observed following TAA-treatment for 6 weeks that may result from the initial tissue homeostatic response to increased lipid peroxidation and as a compensatory mechanism against tissue damage.

Conclusion

In conclusion, our study shows that administration of thioacetamide for 18 weeks induces liver cirrhosis accompanied with oxidative stress imbalance in cirrhotic rats' brain tissues. The decrease in the mitochondrial complexes activities with general decline in the antioxidant system efficiency contribute to the elevation of reactive oxygen species levels and consequently increased lipid peroxidation in brain cells. Therefore we suggest that altered mitochondrial complexes I and III is implicated in TAA-induced hepatic encephalopathy. More studies are required to explore the molecular pathogenesis of mitochondrial implication in hepatic encephalopathy and possible way to prevent it.

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