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Chronic Liver Failure Induces Oxidative Stress and Mitochondrial Dysfunction in Rat Hippocampus

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Abstract: **Brain functions are intricately linked to the proper functioning of the liver such that compromised liver function led buildup of neurotoxins, like ammonia, in the cerebral circulation is argued to derange brain functions in many ways. The neuronal damage during chronic liver failure (CLF) seems to be associated with oxidative stress and bioenergetic derangements. We have attempted to examine the level of oxidative stress markers and mitochondrial dysfunction in the hippocampus of the rats with CLF induced by administration of 100 mg/Kg bw thioacetamide (TAA).; ip. As compared to the control group rats, the CLF rats showed declined levels of the key antioxidant enzymes like superoxide dismutase, glutathione peroxidase and small molecule antioxidant glutathione in their hippocampus. This was consistent with the enhanced level of lipid peroxidation and protein carbonyl content. The significantly declined level of cellular ATP, the enhanced mitochondrial ROS level and release of mitochondrial cytochrome c in the hippocampus further suggested deranged mitochondrial function in this brain region of the CLF rats. The findings suggest imposition of oxidative stress and mitochondrial dysfunction in the hippocampus due to CLF in rats.**

Index Terms: **Chronic liver failure, brain derangement, hippocampus, mitochondrial dysfunction, oxidative stress**

I. INTRODUCTION

Chronic liver failure (CLF) leads to a serious metabolic brain disorder, characterized by multifactorial neurological derangements, which is known as Hepatic Encephalopathy (HE). This is a symptomatically reversible disorder, which affects approximately 30 -50% of patients with cirrhosis (Butterworth, 2019). It is argued that multiple mechanisms could be involved in the pathogenesis of HE. As moderate level of HE is characterized mainly by impaired memory functions (Khanna et al., 2020), it is advocated that the brain region specific neurochemical changes might be playing important roles in HE pathogenesis and therefore targeting hippocampus, accountable for memory formation, needs special attention for investigation

When liver function is compromised, the detoxification of ammonia to urea is impaired resulting into accumulation of ammonia in the blood, a condition called hyperammonemia (HA), in the patient. Consequently, this enhanced serum ammonia diffuses across the BBB and reaches to brain thereby causing brain edema and neuroinflammation followed by derangement of a number of neurochemical aberrations in the brain cells (Sureka et al., 2015). The most plausible mechanism of neuropathology associated with HE is the mobilization of ammonia into glutamate resulting into increased level of this excitatory neurotransmitter in the synaptic cleft. Persistent exposure to accumulated glutamate causes overactivation of postsynaptic glutamate receptors, specifically ionotropic receptors, at downstream level, leading to neurochemical aberrations associated with the development of neuropsychiatric complications (Felipo, 2009; Sureka et al., 2015). Although the exact neurochemical basis of pathology associated with CLF led encephalopathy remains elusive, the overactivation of ionotropic excitatory receptor led deranged metabolic functions (declined cellular ATP) and enhanced ROS accumulation are argued to be responsible for neurodegeneration and neuroexcitotoxic changes during HE pathogenesis (Butterworth 2019, Rao & Norenberg, 2012).

Since the brain is highly dependent on mitochondrial ATP production, the association between mitochondria and development of metabolic brain disorder has been suggested by a number of studies. Mitochondrial functions are inseparable in terms of ATP production and maintenance of oxidative balance, however, ammonia induced disruption of mitochondrial function could be a contributing factor for the neuronal derangement in case of HA pathogenesis as well (Rao & Norenberg, 2012; Heidari, 2019). The hyperammonemia led inhibition of mitochondrial function leads to disproportionate ROS generation which severely affects the cellular homeostasis and causes cellular damage. Therefore, oxidative stress could be another vital player in the pathophysiology of the ammonia induced neuronal injury (Heidari, 2019).

The mechanistic connection between mitochondrial dysfunction and oxidative stress are found to be associated with defect in primary antioxidant defense molecules like SOD, GPx and thiol. It has been suggested that the activity of enzymes like superoxide dismutase, glutathione peroxidase and synthesis of glutathione has been suppressed during HE pathogenesis (Heidari, 2019).

In order to examine the status of oxidative stress markers and mitochondrial bioenergetics specifically in the hippocampus (accountable for the memory function), this study, therefore, focuses on the evaluation of extent of CLF led oxidative stress and mitochondrial dysfunction in the hippocampus of the chronic liver failure rats.

II. MATERIALS & METHODS

A. Animal

All the experiments were performed with the adult male albino rats weighing 160-180g; rats were kept in separate cages and fed with the recommended diet, also were maintained on a standard 12:12 h light: dark cycle, as per recommendations of the Institutional animal ethical committee.

B. Development of chronic liver failure (CLF) Model

The chronic liver failure model of neuronal derangement in adult male albino rats was developed by the administration of hepatotoxin, thioacetamide (TAA) as standardized in our lab (Singh & Trigun, 2010; Khanna et al., 2020). Briefly, the rats were divided into two groups (n=6) Control group administered with 0.9% NaCl i.p, once daily for 10 days; Chronic Liver Failure (CLF) group were injected with 100 mg/kg bw TAA (prepared in 0.9 % NaCl) i.p once daily for 10 days. After 24h of the last dose given, all the rats were sacrificed and the hippocampal tissue was isolated for further biochemical studies. The hyperammonemic condition was assessed by liver function test and ammonia assay as reported earlier (Singh & Trigun, 2010).

C. Preparation of cytosolic extract

Hippocampus extracts were prepared using 0.02 M Tris–Cl (pH 7.4) buffer containing protease inhibitors by using the Potter Elvehjem homogenizer fitted with Teflon pestle. Extracts were centrifuged at 15,000 x g for 45 min at 4°C, the supernatant was collected as crude cytosolic extract used for ammonia assay, oxidative stress parameters. Protein content was estimated by the Lowry et al., (1951) method.

D. Preparation of mitochondrial extract

As reported earlier (Anamika & Trigun, 2021), the mitochondrial extract was prepared in ice cold MSH homogenization buffer (225 mM Mannitol, 75mM Sucrose, 1mM EGTA, 10mM HEPES (pH 7.2) and 1% bovine serum albumin. The tissue was homogenised in MSH buffer and was centrifuged at 1000 x g for 5 min to remove cell debris. The supernatant was collected and re-centrifuged at 10,000 x g for 10min to isolate a crude mitochondrial pellet. The pellet was further washed twice with MSH buffer without EGTA and the pellet so obtained was re-suspended in the MSH buffer without EGTA. Protein content was estimated by the Lowry et al., (1951) method.

E. Lipid peroxidation

The amount of malondialdehyde (MDA), the product of lipid peroxidation, was measured using the method described earlier (Placer et al., 1966). The method measured the trimethionine (pink colour) formed by the reaction of 1mole of MDA and 2 mole of thiobarbituric acid (TBA) in an acidic medium. The spectrophotometric absorption of trimethionine formed was measured at 548 nm. The procedure can be briefly described, 1 mL of Tris-Maleate buffer (0.2 M, pH 5.9) and 0.5 mL sample was incubated at 37ºC for 30 min. Thereafter, 1.5 mL of TBA was added to the mixture and was placed in boiling water bath for 10 min. The sample was then allowed to cool down at room temperature, and was then added with 3 mL of pyridine: n-Butanol mixture (3:1 v/v) and 1mL of 1 N (w/v) NaOH. The contents were thoroughly mixed and allowed to stand for 10 min at RT. The absorbance was read at 548 nm and the levels of lipid peroxidation were expressed as nmole MDA/ g wet wt.

F. Protein carbonylation

The method for Protein carbonyl content estimation was adapted from Siqueira et al., (2005). Briefly, the sample were incubated with 1% streptomycin sulfate solution for 15min. and was then centrifuged at 3600 x g, the supernatant was collected and divided into two parts: one part is incubate with

10 mM DNPH in 2 M HCl and other part with 2 M HCl (considered as blank) for 1h at room temperature. The incubated mixture was then precipitated by adding equal volume of 20% TCA, followed by centrifugation at 8600g. The pellet obtained was washed three times with ethanol: ethyl acetate (1:1) to remove excess DNPH. The precipitate obtained was dissolved in 6M guanidine HCl and optical density was measured at 370 nm. Carbonyl content was measured as nmol/mg of protein.

G. Mitochondrial ROS estimation by DCFDA metho:

2', 7' –dichlorofluorescindiacetate (DCFDA) is a fluoroscent dye that measures hydroxyl, peroxyl and other reactive oxygen species (ROS) within the cell. In presence of ROS, DCFDA is converted into a highly fluorescent compound, 2', 7' –dichlorofluorescein (DCF) by cellular esterases. DCF is then detected by fluorescence spectroscopy with maximum excitation of 485nm and emission spectra of 529nm. Briefly, freshly prepared crude cytosolic and mitochondrial fractions equivalent to 50µg protein were incubated with DCFDA (100µM) for 30 min at room temperature. Amount of ROS was quantified at excitation and emission wavelength of 485nm and 529 nm, respectively. The amount of ROS was expressed as relative fluorescent intensity/mg protein.

H. Estimation of thiol content

The spectrophotometric method for estimation of thiol is based on the Ellman's method which uses 5-5'-dithiobis [2-nitrobenzoic acid] DTNB. The procedure involves the reaction of ellman's reagent with the sulfahydryl group to form 5-thionitrobenzoic acid (TNB) and GS-TNB chromophore, which is quantified by measuring the absorbance at 412 nm as described earlier (Mehrotra nd Trigun, 2012). Briefly, 25µl of fresh mitochondrial extract was added with 75 µl dilution buffer (30mM Tris-Cl, 3mM EDTA pH8.2) and was thoroughly mixed. Thereafter, 20 µl DTNB solution (15.18 mg DTNB/5ml methanol) and 400µl of methanol was added into it and again mixed. The sample was spun at 3,000g for 5 min at room temperature and the supernatant was transferred to a multiwell microplate reader and absorbance was read at 412nm. For quantification of free thiol, the fresh homogenate was subjected to the protein precipitation using 5% TCA (double the sample volume) followed by centrifugation at 1500rpm for 15min. The supernatant collected was mixed with dilution buffer (262mM Tris-Cl and 13mM EDTA, pH 8.9) and absorbance was read at 412nm. Using molar extinction co-efficient of 13,600 M-1cm-1 for the DTNB was used for calculation and values were expressed as nmol/mg protein.

Calculation was done as:

Total SH concentration (µM) = [(Total volume/sample volume) X OD at412nm]/1360

I. Analysis of SOD and GPx by non-denaturing PAGE

Non-denaturing polyacrylamide gel electrophoresis was done as already described (Mehrotra& Trigun, 2012). 60 µg protein of hippocampus tissue extracts of control and experimental rats were loaded on non-denaturing polyacrylamide gel and electrophoresis was carried out at 4ºC at constant voltage (100V) for 2h. The gels were then incubated in 20 mL activity staining mixture at 37ºC for 15-20 min in staining solution. For SOD activity staining, the gel was soaked in a solution of 0.25mM NBT, 28µM Riboflavin and 28mM TEMED. NBT (Nitro Blue Tetrazolium) is used as a competitor for O_2 radical and colour indicator. NBT and SOD in the gel compete for O_2 radical such that the SOD activity zone appeared transparent, while rest of the region became purple blue due to reduced NBT. For Glutathion peroxidise (Gpx): the gel was submerged in 50 mM TrisCl buffer (pH 7.9) containing 13mM reduced glutathione (GSH) and 0.003% hydrogen peroxide with gentle shaking for 30 min. The Gpx activity was stained with a solution containing 1.2mM NBT and 1.6mM PMS for 10 min. The clear band against the purple background was recorded as the active Gpx bands. After development of activity bands, the gels were photographed and intensity of bands was quantified by gel densitometry using Alphaimager 2200 gel documentation software.

J. Measurement of cellular ATP level

The ATP content in the hippocampal tissue extract was measured as per manufacturer's instruction provided in ATP assay kit purchased from Abcam. The assay was based on the simple principle where phosphorylation of glycerol was utilised to produce a spectrophotometrically active compound which is quantified at 570nm. Briefly the fresh tissue was homogenized in the ATP assay buffer provided in the kit. The homogenate was centrifuged at 15000g for 5 min. to pellet down insoluble debris. The supernatant was then mixed with ATP probe, ATP converter, followed by addition of developer mixture. The content was thoroughly mixed and was incubated in the dark for 30 min. The optical density was then measured at 570nm. The ATP content was expressed as picomoles/mg protein.

K. Western Blotting

Briefly, cytosolic extract equivalent to 80µg protein was loaded onto 15% denaturing polyacrylamide gel and electrophoresis was carried out at constant voltage of 100 volts for 2 hours followed by transferring the protein bands on nitrocellulose membrane at 35mA for 2hrs at 4 °C. Efficiency of protein transfer was assessed by Ponceau S staining. To avoid non-specific antibody signal, blocking of the membrane was

done in blocking solution (5% non-fat dried milk prepared in 1X PBS) for 90min. The membrane was then incubated with primary antibody anti- Cyt c (1:500 dilution), prepared in blocking solution and was kept on a rocker-shaker at 4ºC overnight. HRP-conjugated secondary antibody was used for final immunodetection using ECL western blotting detection kit. For loading control, monoclonal anti-β-actin antibody (1:10,000 dilution) was used. The normalized densitometric values of Cyt c vs β-actin, was recorded using gel densitometry software Alpha Imager 2200.

L. Statistical analysis

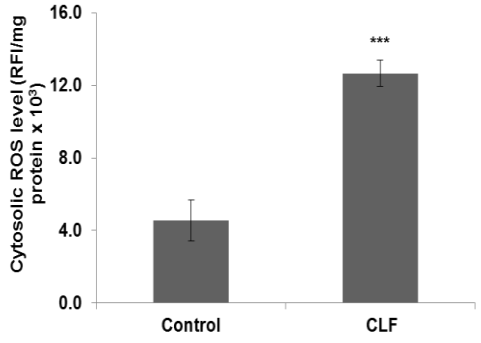
The statistical analysis of the data obtained was done by unpaired Student's t test, and the result is expressed as mean ± SD and $p<0.05$ was taken as the level of significance between the control and experimental sets.

III. RESULTS

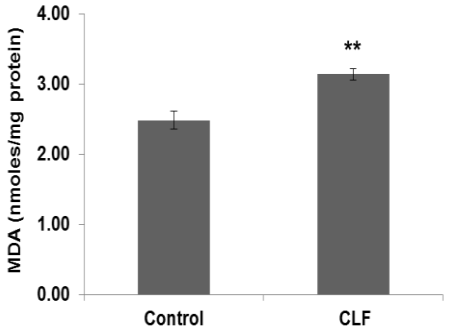
A. *Enhanced levels of oxidative stress markers in hippocampus of the CLF rats*

The oxidative stress, measured as reactive oxygen species level using DCFDA method, showed that in comparison to the control rats, total ROS was significantly higher in the hippocampus ($p<0.001$) of the CLF rats (Fig. 1A). In agreement to the enhanced ROS accumulation, the lipid peroxides measured as the MDA level (Fig. 1B) and the protein carbonyl level (Fig. 1C) was also found to be significantly increased in the hippocampus ($p<0.01$) These findings, thus suggests for a pro-oxidant condition at cellular level, in hippocampus of CLF rats.

(A)



(B)



(C)

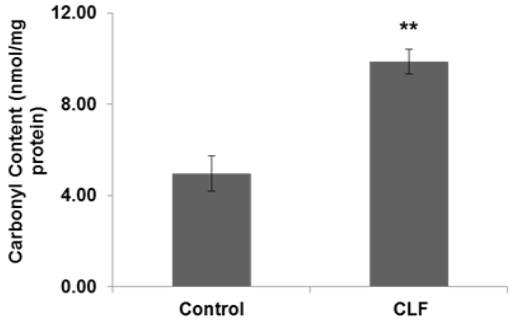


Fig. 1: *Level of ROS estimated by DCFDA assay (A); Level of MDA (B); Level of carbonyl content (C) in the hippocampus of control and CLF rats. Values are represented as mean ± SD; n=5; **p<0.01, ***p<0.001(control vs CLF rats).*

The enhanced oxidative stress suggests for a derangement in the antioxidant defense system and therefore, next we assessed the activity and level of antioxidant enzymes. Superoxide dismutase (SOD) is the committed enzyme that forms the first line defense in the central antioxidant pathway by neutralizing the free oxygen radical to H_2O_2 which is further metabolized by GPx into water and oxygen. The GPx catalysis requires thiol and this small molecule serves as antioxidant buffering system and thereby it is involved in maintaining cellular redox status. The active levels of these enzymes were analyzed by non-denaturing PAGE and the data obtained suggests that the active levels of SOD (Fig. 2A) and GPx (Fig. 2B) is found to be significantly declined ($p<0.001$) in the hippocampus CLF rats as compared to the control group. A similar trend was observed in case of the cellular thiol (free and total) content which was found to be significantly declined in the hippocampus of CLF rats (Fig. 2C).

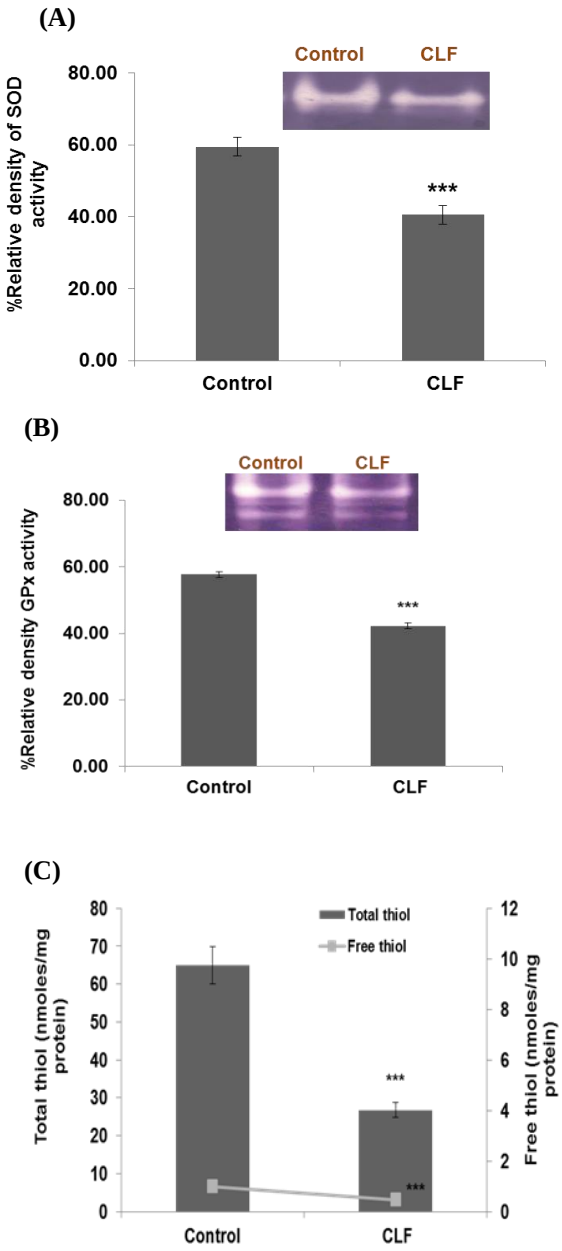


Fig. 2: *In gel activity assay of SOD (A); GPx (B) and level of thiols (C) in hippocampus of the control and CLF rats. The representative photographs from five PAGE repeats are presented with their densitometric values presented as mean ± SD; n=5; ***p<0.001(control vs CLF rats).*

B. Declined Energy status in the hippocampus of the CLF rats

As such, persistent enhanced ROS level is argued to be associated with the compromised mitochondrial function and energy deficit in most of the brain regions. In agreement with this, as shown in Fig. 3, we found a significant decline in the cellular ATP level in the hippocampus (p<0.001) of CLF rats in comparison to the control group rats.

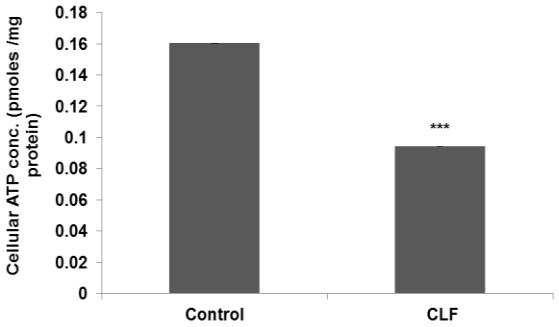
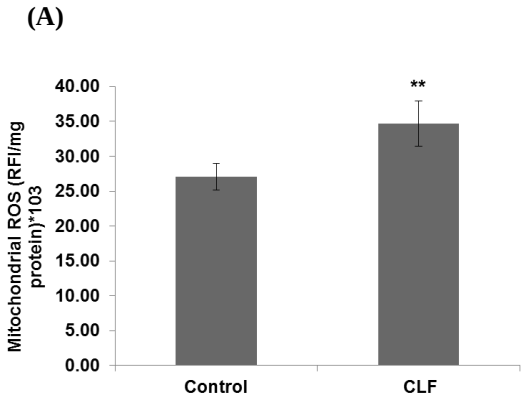


Fig. 3: *Level of ATP in hippocampus of control and CLF rats. Values are represented as mean ± SD; n=5; ***p<0.001(control vs CLF rats).*

C. *Enhanced level of mitochondrial ROS and disruption of mitochondrial membrane in the hippocampus of the CLF rats*

Mitochondria are the main site of energy production and consequently the main producer of the highest reactive oxygen species. Enhanced mitochondrial ROS level is thus, suggestive of increased electron leakage from the electron carriers as a result of mitochondrial dysfunction. As shown in Fig. 4(A&B), in comparison to the control group rats, there was significantly increased ROS level and the extent of protein oxidation as measured by mitochondrial carbonyl content in the mitochondria of the hippocampus (p<0.01).



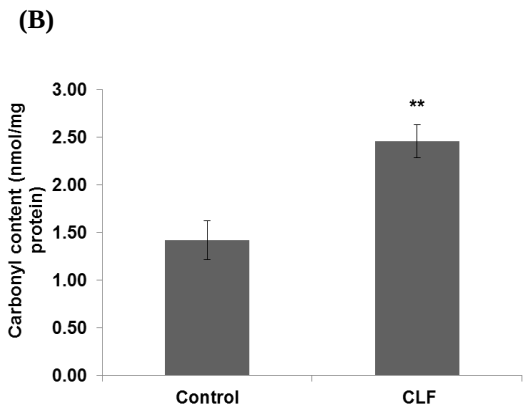


Fig. 4: *Mitochondrial ROS generation assayed by DCFDA (A) & protein carbonyl content (B) in the hippocampus Control and CLF rats. Values are represented as mean±SD; n=5; **p<0.01(control vs CLF rats).*

The enhanced mitochondrial ROS load *vis a vis* compromised bioenergetic activity is likely to result into release of the cytochrome c from this organelle. The release of cytochrome c from mitochondria is considered the rooting step for the mitochondrial dysfunction led apoptosis. As shown in the Fig. 4(C) it was observed that cyt c content in the cytosol increases significantly in the hippocampus (p<0.01) of the CLF rats as compared to the control group of rats.

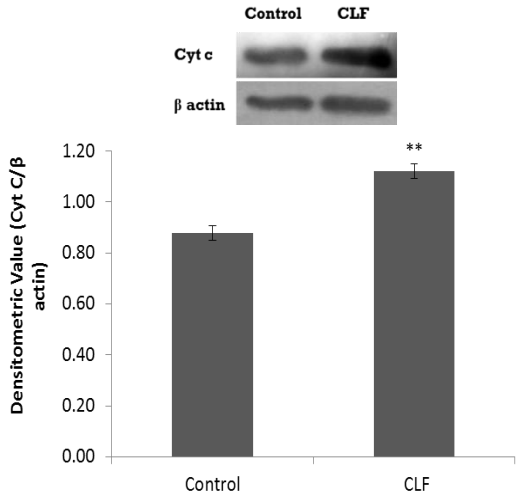


Fig. 4(C): *Profile of cytochrome c in hippocampus of control and CLF rats. Values are represented as mean±SD; n=5; **p<0.01 *p<0.05 (control vs CLF rats).*

IV. Discussion

A diseased liver often results in the onset of psychomotor and cognitive impairment, along with severe personality changes which can develop into coma. The severity of brain injury is associated with the degree of liver dysfunction such that liver failure is directly correlated to the rise in systemic ammonia concentration, a condition known as hyperammonemia, causing neuronal injury in the CNS. Ammonia is a known neurotoxin which causes brain edema, neuroinflammation, mitochondrial dysfunction, disrupted energy metabolism and oxidative stress. Ammonia adversely affects the mitochondrial stability altering mitochondrial permeability transition which mediates the molecular signaling ultimately causing cell death (Heidari et al., 2019). Thus, the present study investigated the effect of chronic liver failure on oxidative parameters and mitochondrial function in the hippocampus, the brain region involved in high order brain functions like cognition and memory formation.

The enhanced level of ammonia in the brain is swiftly metabolized to produce an excitatory neurotransmitter glutamate as a result of concordant activities of the two key enzymes: Glutamine synthetase (GS) and Glutaminase (Glnase), present in astrocytes and neurons respectively (Felipo, 2009). There are a number of reports suggesting that hyperammonemia and hepatic failure result in higher level of extracellular glutamate in the brain resulting into the altered glutamatergic neurotransmission and over activation of glutamate receptors which is now considered as the main mechanism of glutamate excitotoxicity (Lau &Tymianski, 2010). Impaired glutamatergic neurotransmission is likely to cause neurodegeneration by involving enhanced ROS generation, lipid peroxidation of the cell membrane, protein carbonylation, bioenergetic deficit and mitochondrial derangement (Lin & Beal, 2006). In this context, we have also reported earlier that chronic liver failure leads to elevated serum and brain ammonia level vis a vis concordant alteration in brain physiological state (Singh & Trigun, 2010; Khanna & Trigun, 2016; Anamika & Trigun, 2021). In the present report, significant increases in the cellular ROS, lipid peroxidation and protein oxidation level, Fig. 1 A, B & C, clearly suggest for the involvement of oxidative stress and altered redox homeostasis in the hippocampus of the CLF rats.

With high oxygen demand and polyunsaturated fatty acid enriched myelinated nerve fibres, brain is highly susceptible to ROS insult and therefore, it has an effective antioxidant defense system. In the present context, we also studied the level of antioxidant enzyme SOD, the first enzyme in the antioxidant defense line which converts $O_2^{\bullet-}$ into H_2O_2 which is further efficiently removed by either catalase or GPx and thereby protecting brain cells from the oxidative damage. Glutathione forms the major small molecule antioxidant defense mechanism in support of the SOD-GPx/Cat system (Lin & Beal, 2006). Therefore, the findings from Fig. 2A (SOD level), Fig. 2B; GPx level and the glutathione level (Fig. 2C) .suggest a concordant decline in the antioxidant defense system in the hippocampus of the CLF rats and thereby arguing for involvement of oxidative stress in the CLF led brain pathogenesis.

Abnormalities in hyperammonemia led glutamate metabolism provide a critical nexus to the etiology of neurological derangement during persistent liver disease, represented in terms of metabolic and bioenergetic failure. Levels of high energy metabolites such as ATP and phosphocreatine have been reported to decrease in several animal models of chronic liver failure led metabolic brain disorder including HE (Felipo, 2009). The reduction in brain ATP content during disease progression may be due to declined oxidative phosphorylation and mitochondrial function or may be due to the increased energy expenditure (Lemberg & Fernandez, 2009; Heidari, 2019). As we observed marked decline in cellular ATP level (Fig. 3) coinciding with a previously reported similar decline of ATP/AMP ratio in the mitochondria (Anamika & Trigun, 2021), it is argued that the enhanced energy expenditure might be accountable for the observed bioenergetic deficit in the hippocampus during CLF.

Since brain is a high energy demanding tissue, its dependence on mitochondria is higher and therefore, the mitochondrial dysfunction has a major impact on neuronal homeostasis and brain functioning. Deranged mitochondrial function often emerges with declined oxidative phosphorylation and enhanced reactive oxygen species (ROS) generation because of increased electron leakage through different electron carriers. The data from the present study suggests that the impaired cerebral energy metabolism (Fig. 3) is in agreement with increased mitochondrial ROS level and oxidative stress as assessed by mitochondrial protein carbonyl content (Fig. 4 A&B), which contributes majorly to the increased loss of mitochondrial function.

The increased ROS generation and impaired energy metabolism is likely to cause altered mitochondrial membrane permeability transition. mPT (mitochondrial permeability transition) is a sudden change in the inner mitochondrial membrane permeability which facilitates movement of small molecules and ultimately loss of membrane potential. Collapse of mitochondrial membrane potential leads to osmotic swelling of the mitochondrial matrix and release of not only the apoptotic factors but also necrotic factors (Wang et al., 2019). In the present study, a similar observation of enhanced ROS level and declined mitochondrial function suggest about the declined mitochondrial potential leading into the release of cytochrome c in the cytosol (Fig. 4C).

Cyt c is a peripheral protein found in mitochondrial inner membrane where it functions as an electron carrier between respiratory chain complex III and complex IV. Cytochrome c is usually tightly restricted to the inner membrane, but once released to the cytosol, it initiates the apoptotic cascade and challenges the neuronal survival (Portt et al., 2011). A similar study in animal MHE (Minimal Hepatic Encephalopathy) model has shown that mitochondrial dysfunction is associated with the astrocyte death, where the enhanced Bax/Bcl2 ratio, loss of membrane potential and cyto c release have been argued indicative of induction of apoptosis in the brain (Bustamante et al., 2011).

CONCLUSION

The Chronic Liver Failure developed by injection of thioacetamide is likely to affect brain adversely mainly by imposing oxidative stress which could be attributed to the CLF led declined level of antioxidant factors in this brain region. The imbalanced cellular redox status thus generated could be associated with the concordantly impaired mitochondrial bioenergetics in the hippocampus of those CLF rats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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