CEREBRAL MITOCHONDRIAL DYSFUNCTIONS IN THE ETIOLOGY OF THIOACETAMIDE INDUCED FULMINANT HEPATIC FAILURE

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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Dedicated
to
my beloved parents

University of Hyderabad

(A Central University established in 1974 by act of parliament)

HYDERABAD - 500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled ***"Cerebral mitochondrial dysfunctions in the etiology of thioacetamide induced fulminant hepatic failure"*** has been carried out by me under the supervision of Late Prof. Ch.R.K. Murthy and later under Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

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ABBREVIATIONS

AST	: Aspartate Aminotransferase
ADP	: Adenosine 5'-Di phosphate
ALT	: Alanine Aminotransferase
BSA	: Bovine Serum Albumin
CoQ	: Ubiquinone
DAM	: Di Acetyl Monoxime
DCPIP	: Dichlorophenol Indophenol
DTNB	: Dithio Nitro Benzoic acid
EDTA	: Ethylene Diamine Tetra Acetic acid
FAD	: Flavin Adenine Dinucleotide
$FADH_2$	: Flavin Adenine Dinucleotide reduced
FHF	: Fulminant Hepatic Failure
g	: Gram
GABA	: Gamma Amino Butyric Acid
GAD	: Glutamic Acid Decarboxylase
GOD	: Glucose Oxidase
GSH	: Glutathione reduced
GSSG	: Glutathione oxidized
h	: Hour(s)
HE	: Hepatic Encephalopathy
HEPES	: (N-[2-Hydroxyethyl]piperizine-N-2-[ethanesulphonic acid])
INT	: 2-(4-Iodophenyl)-3-(4-Nitrophenyl)-5-phenyl tetrazolium chloride
MDA	: Malondialdehyde
mg	: Milligram
min	: Minutes
ml	: Milliliter
mM	: Millimolar
NAD	: Nicotinamide Adenine Dinucleotide
NADH	: Nicotinamide Adenine Dinucleotide reduced
NADP	: Nicotinamide Adenine Dinucleotide Phosphate

NADPH	: Nicotinamide Adenine Dinucleotide Phosphate reduced
NBT	: Nitro Blue Tetrazolium
nm	: Nanometers
NMDA	: N-methyl-D-aspartate
OAA	: Oxaloacetate
OPT	: O-pthalaldehyde
P/O	: Phosphate to Oxygen Ratio
PMS	: Phenazine Methosulfate
RCR	: Respiratory Control Ratio
SDH	: Succinate Dehydrogenase
SOD	: Super Oxide Dismutase
TAA	: Thioacetamide
TCA	: Tricarboxylic acid cycle
UV	: Ultraviolet
α-KG	: α-Ketoglutarate
μM	: Micromolar
ROS	: Reactive Oxygen Species
NEM	: N- Ethyl Maleimide
TEM	: Transmission Electron Microscopy

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Introduction

1.1 Hepatic Encephalopathy

Hepatic encephalopathy (HE) is a clinical syndrome characterized by a number of neurological, neuropsychiatric and motor disturbances seen in patients with liver dysfunction. HE is classified into two major divisions, acute (fulminant) and chronic based on the type of liver dysfunction. Fulminant hepatic failure (FHF) is a clinical syndrome resulting from severe inflammatory or necrotic liver damage. FHF is rapid in its onset and the symptoms appear within a short period of time. This is associated with sudden onset of necrosis of hepatocytes and degeneration of liver tissue without any established liver disease (Katelaris *et al.,* 1989; Sherlock *et al.,* 1971). Chronic hepatic encephalopathy, on the other hand, is slow in its occurrence and it takes a long period of time before the symptoms appear. The development of this form is gradual and it happens over a long period of time. The present study concentrates on the former type of the two kinds of Hepatic encephalopathy mentioned above.

1.1.1 Symptoms of Fulminant Hepatic Failure (FHF)

A wide spectrum of neurological, neuropsychiatric and neuromuscular derangements are associated with various stages of FHF. In the early stages, very subtle disturbances in the sleep rhythms, personality and emotional changes are observed. As the condition deteriorates, hypothermia, hyperventilation, confusion and drowsiness are seen (Jalan, 2003; Gayed *et al.,* 1987; Margolis *et al.,* 1979; Strauss *et al.,* 1998). Seizures are not uncommon and multifocal

random muscle twitching is often seen before coma (Brown *et al.,* 1992). In addition, neuromuscular changes such as asterexis, hyperflexia, unsustained clonus, deterioration of pupillary, corneal, occulovestibular, occulocephalic and brain stem reflexes are seen. Convulsions, cortical blindness, retarded speech, deterioration of speech, deterioration of EEG, sixth nerve palsy are also seen in these patients (Hoyumpa and Schenker, 1985) which finally lead to coma and death (Kanamori *et al.,* 1996; Dejong *et al.,* 1993). Chronic hepatic failure is slow in its onset and the symptoms appear gradually over a period of time.

1.1.2 FHF- Etiology

An important cause associated with fulminant hepatic damage is acute viral hepatitis. Of the several types of hepatitis viruses, hepatitis B is the most common and accounts for 74% of the clinical cases (Floersheim *et al.,* 1975; Saunders *et al.,* 1972). Non-A, non-B type viral hepatitis accounts for 24%, while type A accounts for 2% (Papas-venegalu *et al.,* 1984). Because of the horizontal and vertical spread of virus, this viral infection often assumes epidemic proportions.

Next to viral infections, ingestion of hepatotoxins also result in FHF. These include indiscriminate usage of drugs such as paracetamol (acetaminophen) (Morton *et al.,* 1999; Baudouin *et al.,* 1995), halothane (Braude *et al.,* 1981), tetracycline, valproic acid (Ziyeh *et al.,* 2003), anti-tuberculosis drugs, sulfonamides, diuretics etc (Plum and Hindfelt, 1976; Papas-Venegelu, 1984). These drugs, especially

paracetamol, act as hepatotoxins when they are used indiscriminately in large doses (Braude *et al.,* 1981). Food contaminants, such as aflotoxins (fungal contaminants on ground nut), mushroom poisons, shellfish poisons, bacterial toxins and heavy metals also cause massive necrosis of hepatocytes and FHF (Mullen *et al.,* 1994; Hoyumpa *et al.,* 1985; Papas-Venegalu *et al.,* 1984; Braude *et al.,* 1981; DeLong *et al.,* 1982; Burroughs *et al.,* 1982). Other known causes for FHF are Wilson's disease and hepatic malignancy (DeLong, and Glick, 1982; Burroughs *et al.,* 1982). FHF may also be due to extensive accumulation of fat in the liver as in Reye's syndrome, in pregnancy, and in patients with surgically constructed jejunoileal bypass for morbid obesity (Burroughs *et al.,* 1982; Delong and Glick, 1982).

In hepatic failure surveillance study, a very poor survival rate (22%) was observed in the patients. Clinical outcome of the patients with FHF depends on the number of surviving hepatocytes, age, sex, etiology and the stage at which the patient is provided with medical help. There is no specific treatment or drugs to be administered to the patients with Fulminant Hepatic Failure and the outcome depends on the supportive intensive care and better patient management. Even then, management of FHF patients is less rewarding (Hoyumpa and Schenker, 1985; Ferenci, 1991; Mullen *et al.,* 1994).

1.1.3 Management of Patients

Various therapeutic procedures are adopted to treat Fulminant Hepatic Failure with limited success. These include dietary restrictions, administration of lactulose, lactulose + neomycin Sulfate, infusion of synthetic mixtures of amino acid or a-keto acids (Iwasa *et al.,* Butterworth, 2003), dialysis (including haemodialysis, peritoneal dialysis), providing artificial liver support (by way of extracorporeal dialysis, administration of fetal liver cell agglutinates etc), use of bioreactors with immobilized hepatocytes and finally liver transplantation. The rationale behind these therapeutic approaches, their advantages and disadvantages are not discussed here as it is out of the scope of present investigation. However, it is suffice to mention here that the outcome of these therapeutic practices is not very satisfactory and the results are equivocal. This is quite evident from the poor survival rate (22%) of these patients and the final out come is chiefly dependent on the support and management of the patient (Gerlach *et al.,* 1994; Sussman *et al.,* 1993; Takabatake *et al.,* 1991; Morgan *et al.,* 1990; Cooper *et al.,* 1987; Conn *et al.,* 1979).

Multiplicity of the therapeutic practices and the uncertainities in their outcome in FHF might be due to the enigmatic nature of the pathophysiological mechanisms involved in this condition. Several mechanisms have been proposed to explain the pathophysiology of FHF. Of these, only the major ones are described below. However, it is also cautioned that

- these are not the ultimate mechanisms
- there may be some other mechanism(s) to be discovered
- more than one mechanism may be acting synergistically for the neural dysfunction in FHF and finally
- there could even be a unified mechanism.

The uncertainty of enigmatic mechanism of the pathophysiology of the disorder is also attributed to

- Lack of an appropriate animal model, which closely mimics the clinical condition of the human disease.
- Regional cellular heterogeneity of the brain which renders the studies more difficult, if not impossible. This heterogeneity in brain is seen in structure, function, and metabolism at cellular and sub cellular levels of organization.
- Multiplicity of factors implicated in the etiology of cerebral dysfunction in HE has further complicated the issue. These factors include ammonia, false neurotransmitters, mercaptans and short chain fatty acids (Zieve, 1981).

1.1.4 Pathophysiological Mechanisms

1.1.4.1 Ammonia

Elevated level of blood and tissue (including brain) ammonia is the hallmark of conditions of hepatic inadequacy (Jayakumar *et al.,* 2004; Jalan *et al.,* 2003; Murthy *et al.,* 2000; Norenberg *et al.,* 1996; Albrecht 1998; Ferenci *et al.,* 1991; Hoyumpa *et al.,* 1985; Cooper *et al.,* 1987). Intestine is the major site for the production of ammonia in

the body. The microbial flora of the intestine act on the dietary nitrogenous compounds and produce large quantities of ammonia. Moreover, urea, which reaches the gut through entero-hepatic circulation is used by the microbes to produce ammonia. In addition, intestinal smooth muscle uses glutamine for its energy metabolism and thus generates ammonia even in the post-prondial period. Ammonia, thus generated, enters the portal circulation and reaches liver. In normal conditions, ammonia is efficiently detoxified in the liver by incorporation of ammonia into urea (periportal hepatocytes) and glutamine (perivenous hepatocytes). However, in the absence of functional hepatocytes, ammonia directly enters the systemic circulation and floods all the extra-hepatic tissues including brain. As a result, the levels of ammonia in brain and cerebrospinal fluid are elevated in fulminant hepatic failure and other liver diseases which is known to be neurotoxic (Schenker *et al.,* 1974; Conn *et al.,* 1979). Elevated levels of ammonia in blood and brain have also been reported in several conditions - for example in conditions of congenital deficiencies of urea cycle enzymes (Cooper *et al.,* 1987).

Ammonia exists in two forms - either as unprotonated NH_3 (non-ionized) form or a protonated (ionic) NH_4^+ ion. These two forms are in a state of dynamic equilibrium in a solution and their inter conversion was found to be rapid and time dependent on the pH of the medium. The unprotonated form has a capacity to freely diffuse across the cell membranes as it is lipid soluble (Cooper *et al.,* 1987; Roos *et al.,*

1981). The protonated NH_4^+ form, on the other hand is largely impermeable. At physiological pH (7.4) about 97-98% of ammonia is in ionic form which is impermeable across the biological membranes. When the pH is towards the alkaline side, large amount of ammonia is present in the freely diffusible form (NH_3) and hence considered to be potentially very toxic.

1.1.4.1.1 Ammonia-Toxicity

The mechanism of ammonia toxicity, by itself, is a subject of intense debate and controversy. Energy depletion theory of Bessman and Bessman (1955) has attracted considerable attention and has been a subject of much controversy. The main tenet of this hypothesis is that ammonia is detoxified in the brain to glutamate and glutamine in the reactions mediated by glutamate dehydrogenase and glutamine synthetase respectively. In the glutamate dehydrogenase reaction, ammonia reacts with α-ketoglutarate to form glutamate with the concomitant conversion of NADH to NAD. This would drain α-ketoglutarate from citric acid cycle and interfere with energy production. Moreover, oxidation of NADH to NAD in this reaction, bypassing electron transport chain, would also affect energy production. In the glutamine synthetase reaction, glutamate reacts with another molecule of ammonia resulting in the production of glutamine and one ATP molecule is used up in this process. It was postulated that a combination of these two reactions would adversely affect the cerebral energy metabolism and consequently the energy dependent metabolic

and physiological processes (Ratnakumari and Murthy, 1993; 1992; 1990; Jessy *et al.,* 1991; Hindfelt and Siesjo, 1971; Bessman and Bessman, 1955). An alternate hypothesis for energy depletion is the adverse effects of ammonia on the operation of malate-aspartate shuttle in the brain (Faff-Michalak *et al.,* 1991; Ratnakumari *et al.,* 1989; Hindfelt *et al.,* 1977). Since glutamine synthesis occurs in the cytosol, it has been postulated that this process depletes cytosolic pool of glutamate leading to lowered cytosolic glutamate. This would enhance the production of lactate and also affect the transport of reducing equivalents across the mitochondrial inner membrane (Therrien *et al.,* 1991; Hindfelt *et al.,* 1977).

Both these hypotheses have been tested vigorously and the results are equivocal - some in favor and some against. However, it is now established beyond doubt that cerebral glutamate levels are decreased while glutamine levels are elevated in the presence of elevated levels of ammonia in the brain (Ratnakumari and Murthy, 1993; 1992; 1990; Jessy *et al.,* 1991; Murthy and Hertz, 1988; Butterworth *et al.,* 1988; Subbalakshmi and Murthy 1985; 1983).

1.1.4.2 Disturbances in Neurotransmitter Functions

Yet another hypothesis proposed for the neurotoxic effects of ammonia is its effect on the neurotransmitter functions. Ammonia, at pathological concentrations, has been shown to interfere with the synthesis, storage (in synaptic vesicles), release and post-synaptic action of major neurotransmitters (Schafer and Jones 1982). As

glutamate and GABA are the chief excitatory and inhibitory neurotransmitters in mammalian brain (Danysz *et al.,* 1995; Fonnum *et al.,* 1984; Ericinska *et al.,* 1990) much of the attention was focused on these two neurotransmitters. Studies from this and other laboratories have shown that in hyperammonemic conditions, glutamate release is enhanced while the reuptake (to terminate the neurotransmitter action) of glutamate is decreased in brain (Rao *et al.,* 1992; 1991). In addition, region specific, time dependent alterations in the binding of glutamate to the receptors, particularly to NMDA subtype, has also been reported in conditions of hyperammonemia with and without liver failure. It is particularly interesting to note that NMDA receptor binding increases in cerebral cortex and pons-medulla regions while there is a great decrease in cerebellum.

Alterations in GABA receptors (parallel and opposite to glutamate), loss of M_1 subtype of muscarinic acetylcholine receptors, receptors for dopamine, serotonin and opioid peptides have also been reported in conditions of fulminant hepatic failure (Rao *et al.,* 1992; 1991; Van der Kloot *et al.,* 1987; Fischer and Baldessarini, 1971).

In addition the involvement of mercaptans (Gracia-Compean *et al.,* 1995; Zeive 1980; Chen *et al.,* 1970; Phear *et al.,* 1956) short chain fatty acids (Butterworth, 2003; Zeive, 1980; samson *et al.,* 1956) have been implicated in the etiology of the cerebral dysfunction in hepatic failure conditions. Reports on hepatic inefficiency conditions also reveal an elevation in the levels of short chain fatty acids. Though the reasons

for this increase in the levels of short chain fatty acids is not understood completely, few investigators report that the decrease in the ATP and creatinine phosphate levels may be the cause (Cooper and Plum 1987; Papas-Venegelelu *et al.,* 1984). In support of this, Zeive *et al* (1974) have observed that the infusion of short chain fatty acids results in the induction of coma.

1.1.4.3 Gamma Amino Butyric Acid (GABA)

GABA hypothesis was proposed by Schaffer and Jones (1982) to explain the HE. Gamma-amino butyric acid (GABA), a neuro inhibitory substance produced in the gastrointestinal tract is believed to be involved in the pathogenesis of HE. About 25%-45% of all the brain nerve endings may be GABAergic. During cirrhosis and FHF an increase in GABA levels is observed (Mullen *et al.,* 1988; Maddison *et al.,* 1987) which is probably due to the decreased metabolism of GABA in liver. When GABA crosses the extra permeable blood brain barrier of cirrhotic patients, it interacts with supersensitive postsynaptic GABA receptors. The GABA receptor, in conjugation with receptors for benzodiazepines and barbiturates regulate a chloride ionophore. Binding of GABA to its receptors permits an influx of chloride ions into the post synaptic neuron, leading to the generation of an inhibitory postsynaptic potential. Administration of benzodiazepines and barbiturates to patients with damaged liver or cirrhosis increases GABAergic tone and predisposes to depressed consciousness. This evidence includes isolation of 1,4-benzodiazepines from brain tissue of

patients with FHF as well as the partial response observed in some patients and experimental animals after administration of flumazenil, a benzodiazepine antagonist (Als-Nielson *et al.,* 2004).

1.1.4.4 Plasma Amino Acids and False Neurotransmitters

Fisher and his group observed an increase in the plasma content of aromatic amino acids (phenylalanine, tyrosine and tryptophan) and a decrease in the content of branched chain amino acids (leucine, valine, isoleucine) in conditions of hepatic failure (James *et al.,* 1979). Observed changes in the plasma content of amino acids in FHF promote the transport of aromatic amino acids from the blood to the brain. Consequently brain will be flooded with aromatic amino acids in the absence of functional liver. As a result of this the content of these aromatic amino acids in the brain will increase beyond the K_m of the respective hydroxylases (phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase) which are rate limiting enzymes in the conversion of aromatic amino acids into their respective neurotransmitter monoamines (dopamine, nor epinephrine, epinephrine and serotonin). In such conditions, aromatic amino acids are decarboxylated directly to aromatic amines such as β-phenyl ethylamine, tyramine, tryptamine *etc,* which are called false neurotransmitters. These amines displace the resident catecholamines (dopamine, nor epinephrine, epinephrine and serotonin) from the synaptic vesicles and are released upon stimulation thus altering the neurotransmitter balance in brain and hence the cerebral function. A

decrease in dopamine and nor epinephrine levels and an accumulation of false neurotransmitters have been reported in conditions of FHF (Ferenci *et al.,* 1984). These false neurotransmitters even bind to the post- and pre- synaptic receptors of biogenic monoamines and switch on otherwise not needed neurotransmission (Fisher and Baldessarini, 1971). This mechanism has been implicated in the etiology of the cerebral dysfunction in conditions of liver inadequacy (Butterworth *et al.,* 1994). Similarly, cerebral levels of serotonin were reported to be increased and the number of serotonin receptors on neurons were observed to be decreased in HE and this might contribute to the neuronal inhibition in HE (Riederer *et al.,* 1982; Cummings *et al.,* 1976). In certain cases of hepatic insufficiency and in experimental animal models, it was observed that perfusion of branched chain amino acids or their keto analogues resulted in an improvement of the clinical condition (Iwasa *et al.,* 2003; Herneth *et al.,* 1998; Beaubernard *et al.,* 1984). It has also been demonstrated in recent years that aromatic amino acids are metabolized by alternate pathways generating kynurenine and quinolinic acid. The latter is known to act as an agonist for NMDA receptors and act as a pro-oxidant (Santamaria *et al.,* 2003a).

1.1.4.5 Benzodiazepines

Apart from the above mentioned factors several groups have reported an increase in the levels of endogenous benzodiazepines leading to their accumulation in conditions of hepatic failure (Jones,

2000; Mullen *et al.,* 1996; Butterworth *et al.,* 1991). Further, an increase in the levels of these compounds also correspond with the progression and severity of the HE (Butterworth 1991; Rothstein *et al.,* 1989). Due to their increased levels, these compounds will bind with the benzodiazepine receptors and trigger the receptors to produce neurosteroids (Papadopoulos *et al.,* 1995) which may play a role in the etiology of disorder (Norenberg, 1997; 1991). An increase in the up-regulation of the peripheral benzodiazepine receptors has been reported by many investigators (Itzhak and Norenberg, 1994; Giguere *et al.,* 1992; Lavoie *et al.,* 1990). This up-regulation has also been shown in hyperammonemic conditions in the cultures treated with pathological concentrations of ammonia (Itzhak and Norenberg, 1994).

The research on the pathology of the brain in conditions of the hepatic failure suggest that the astrocytes have critical role to be played in the etiology of the disorder. Observations of the affected brains have shown astroglial degeneration (Albrecht, 1999, Butterworth, 1998; Norenberg, 1991). Astrocyte swelling is thought to be the major phenomenon that leads to the edema and death of the patients (Vaquero *et al.,* 2003; Cardoba *et al.,* 1996; Swain *et al.,* 1991; Norenberg, 1991; 1977).

1.1.5 Mitochondria - Role in Neurodegenerative Disorders

Mitochondrial dysfunction has been implicated in ischemic brain damage, Alzheimer's disease, Parkinson's disease, Huntington's chorea, Fredric's ataxia, amyotrophic lateral sclerosis, Wilson's

disease, Hereditary spastic paraplegia, some forms of dystonia, Apoptosis/ necrosis, aging, traumatic brain injury and list is still growing (Hatton *et al.,* 2004; Schols *et al.,* 2004; Beal, 2000; Tyurin, 2000; Kroemer *et al.,* 2000; Wallace, 1999; Kaplan, 1999). Recent literature suggests the involvement of mitochondria and oxidative stress in the pathophysiology of FHF.

1.1.5.1 Oxidative Stress

Oxidative stress is a condition in which the production of free radicals is far in excess of their rate of detoxification by endogenous mechanisms (Rao *et al.,* 2002). Being a highly aerobic tissue, accounting for 20% of total oxygen consumed by the body, brain is highly prone to oxidative stress (Gupta *et al.,* 2003). Free radicals are produced in normal course of respiration and are estimated to be about 1 to 2% of the total oxygen consumed by the tissue (Kowaltowski *et al.,* 1999). Furthermore, brain is rich in polyunsaturated fatty acids (Halliwell, 1992) and possesses high content of iron in certain areas, which is supposed to promote free radical production. Added to this, brain has low levels of antioxidant enzymes, low repair mechanisms and non-replicative neuronal cells (Halliwell, 1992). All these factors play a critical role in balancing the damaging effects and the antioxidant defenses. Mitochondria are the major sites of production of free radicals especially by the respiratory electron transport chain (Koch *et al.,* 2004; Fiskum *et al.,* 2004; Muller *et al.,* 2004; Chance *et al.,* 1979). Most of the free radical production occurs in the electron

transport chain present in the mitochondria especially in complexes I and III. Usually, these are detoxified by endogenous free radical scavengers such as glutathione, ascorbic acid and vitamin E and also by the enzymes superoxide dismutase and catalase. Failure of these detoxification mechanisms results in the condition of oxidative stress. It is paradoxical that free radicals affect the function of the complexes of electron transport chain leading to the production of more free radicals (Muller *et al.,* 2004). Moreover, free radicals initiate peroxidation of lipids of the mitochondrial and plasma membrane and also affect the iron-sulfur centers of respiratory chain and enzymes such as aconitase. All these lead to the collapse of mitochondrial membrane potential, altered permeability of mitochondrial membranes and release of cytochrome c and apoptosis initiating factor. The last two activate caspase 3 and initiate the down stream events of apoptotic pathway (Bernardi *et al.,* 1998; Zamzami *et al.,* 1997; Kristal and Dubinsky 1997; Zorratti and Szabo 1995; Gunter and Pfeifer 1990).

1.1.5.2 Mitochondria - Source of Free Radicals

As the power houses of the cell, mitochondria are involved in the generation of ATP through the electron transport system. The transport of electrons through the respiratory complexes is a highly regulated process leading to the flow of electrons from high redox potential to low redox potential compounds. Occasionally some of the electrons are directly transferred to oxygen leading to the production of reactive oxygen species (Cadenas, 2004). In this process instead of two

electrons only one electron is transferred to oxygen resulting in the production of a super oxide anion (Muller *et al.,* 2004). This is highly reactive and unstable oxygen radical. It is postulated that under normal physiological conditions oxygen is converted to superoxide radicals. In addition to this, superoxide radicals are also produced by certain mixed function oxygenases such as xanthine oxidase, aldehyde oxidase, by auto-oxidation of hydroquinones and catecholamines.

The superoxide radicals thus produced can oxidize a variety of biological substances and render them inactive. In addition the superoxide radical also reacts with other compounds to produce other reactive oxygen species. For example super oxide radical reacts with protons to produce hydroperoxy radical which further metabolizes to produce hydrogen peroxide. Superoxide can also react with hydrogen peroxide to produce hydroxyl radicals. In addition superoxide also reacts with nitric oxide to produce peroxynitrite radicals. All these three radicals, like superoxide radical oxidize proteins and lipids and adversely affect their functions. In fact evidences are now accumulating to indicate that free radical production can trigger cell death. Though mitochondria are the primary source of free radical production some amount of free radicals are also generated in other sub-cellular components through a variety of mechanisms. For example: transition metal ions especially iron and copper are known to produce free radicals by Fenton reaction.

1.1.5.3 Antioxidant Defenses

The free radicals that are produced under physiological conditions are detoxified by a variety of antioxidant processes. The cells are endowed with an enzyme superoxide dismutase, which converts superoxide to hydrogen peroxide. Both mitochondrial as well as cytosolic SOD have been identified. The hydrogen peroxide is converted to water and molecular oxygen by the enzyme catalase. It is interesting to note that this enzyme has the highest turnover rate indicating that it can very efficiently nullify the toxic effects of hydrogen peroxide. In addition to these two major mechanisms for detoxification of free radicals, there are several minor pathways participating in this process. For example, the thiol groups of glutathione are oxidized by hydrogen peroxide, there by neutralizing the highly reactive peroxidase. Similarly Ascorbic acid and alpha tocopherol (vitamin E) also participate as antioxidants

1.2 Evidences for Mitochondrial Dysfunctions of Brain in FHF

As the concept of oxidative stress occurring in brain during fulminant hepatic failure is new, there is no direct evidence for this as of today. Some of the results reported earlier for fulminant hepatic failure indicate the conditions which favor increased free radical production in brain during fulminant hepatic failure.

The levels of glutamine are enhanced in the cases of acute and chronic hyperammonemia conditions. (Rao *et al.,* 1992; Butterworth and Giguere, 1986). This increase in the levels of glutamine is due to

the enhanced release and decreased uptake or reduced deamidation of glutamine (Subbalakshmi *et al.,* 1985; 1983; Mathenson and Vandenberg, 1975). In addition to this evidence, Yu *et al.,* (Yu *et al.,* 1984) reported an increase in the synthesis of glutamine in the primary cultures of astrocytes that were subjected to pathophysiological concentrations of ammonia. Decreased uptake of glutamate was observed in primary cultures of astrocytes that were exposed to pathophysiological concentrations of ammonia for four days (Norenberg *et al.,* 1989; 1985). Due to this condition of enhanced release and decrease uptake of glutamate from the nerve terminals, it would result in excessive accumulation of glutamate in the synaptic cleft and in the intracellular spaces in the brain. Glutamate, by itself is the main excitatory neurotransmitter. Prolonged exposure of neurons to glutamate due to the enhanced glutamate release will lead to excessive activation of the receptors for glutamate (especially NMDA subtype) which is supposed to be neurotoxic that leads to neuronal degeneration and cell death (Kosenko *et al.,* 2003). Beal and his group (Beat *et al.,* 1992) have reported that excessive activation of NMDA receptors is involved in neuronal damage in case of ischemic brain and it is also implicated in other neurodegenerative disorders. Glutamate receptors of NMDA subtype are highly permeable to calcium (Ca^{2+}). Elevated NMDA receptor activity results in increased Ca^{2+} influx (Kosenko *et al.,* 1997b; White and Reynolds, 1996) which would hamper the ATP synthesis and thus mitochondrial membrane potential.

This might further result in mitochondrial electron transport chain alterations leading to enhanced production of free radicals and thus resulting in mitochondrial dysfunctions (Nicotera *et al.,* 1997; Choi, 1996). This is supported by the evidence that blockers of NMDA receptors do prevent the glutamate and NMDA neurotoxicity (Kosenko *et al.,* 1999; Koroshetz *et al.,* 1996).

Collapse of mitochondrial membrane potential, production of ROS like superoxide ($O^{2-\cdot}$), NO and activation of several other degradative enzymes are all the events that are proposed to be involved in creating an imbalance of cellular homeostasis that is triggered by the unregulated levels of glutamate (Nikotera *et al.,* 1997; Choi *et al.,* 1996; Beal *et al.,* 1996; Patel *et al.,* 1996; Mattson *et al.,* 1995). A highly reactive peroxynitrite ion may be formed due to the interaction of superoxide and nitric oxide (Hensley *et al.,* 1997). The above discussion suggests that the ROS that are produced due to the increased Ca^{2+} levels have a major role to play in the glutamate mediated toxicity. Mitochondria seem to have an unique role in this regard since it is believed to be the major source of ROS and mobilization of intracellular Ca^{2+} (Ichas *et al.,* 1997; Budd *et al.,* 1996; Bernardi *et al.,* 1994). As discussed above excessive Ca^{2+} accumulation in mitochondria uncouples electron transfer in the electron transport chain, leading to the enhanced production of free radicals (Nikotera *et al.,* 1997; Ichas *et al.,* 1997; Choi *et al.,* 1996). Now mitochondria have emerged as a missing link between the

elevation of Ca^{2+} and ROS mediated glutamate toxicity (Shinder *et al.,* 1996).

Besides the conversion of tryptophan to serotonin, an alternative pathway for tryptophan metabolism has been observed in brain in recent years. In its alternate pathway, tryptophan is converted to kynurenine, kynuramine and kynurenic acid, 3-hydroxy kynurenine and 3- hydroxyl kynuramine. Further conversion of 3-hydroxy kynurenine to 3-hydroxy anthranilic acid and then to quinolinic acid has been detected in brain (Ratnakumari *et al.,* 1993). All these reactions involve the opening of indole ring of tryptophan and the enzymes and the required cofactors are found in brain (Guidetti *et al.,* 1995; Mawal *et al.,* 1991). In addition to the endogenous synthesis, some of these compounds are also transported by neutral amino acids such as leucine, valine, isoleucine tyrosine, tryptophan and methionine (Fukui *et al.,* 1991).

Studies on the formation of these compounds were given importance following the observation that some of them have modulatory effect on the neurotransmission mediated by other neurotransmitters. It has been shown that kynurenine and its derivatives act as antagonists for NMDA receptors while quinolinic acid acts as an agonist (Santamaria *et al.,* 2003a). Quinolinic acid was observed to act as an excitotoxin and prolonged exposure to this compound results in the death of neurons. Kynurenine and its metabolites have been implicated in some of the neurodegenerative

disorders such as ischemic brain damage, Huntington's disease, poliovirus infection, AIDS, septicemia and encephalopathy (Stone *et al.,* 2003; Sardar *et al.,* 1995; Saito ef *al.,* 1993). It has been observed that the content of tryptophan derivatives is enhanced in conditions of fulminant hepatic failure. Hence it has been postulated that kynurenine and its derivatives, especially quinolinic acid might be affecting NMDA receptor mediated functions. As the compounds, which stimulate the glutamate receptors for prolonged period act as excitotoxins, it is believed that excess of quinolinic acid produced under these conditions might be acting as an excitotoxin (santamaria *et al.,* 2003a; 2003b). It is suspected that this might be involved in the Pathophysiology of hepatic encephalopathy in fulminant hepatic failure (Chiarugi *et al.,* 1995). In recent years, it has been shown that quinolinic acid can cause lipid peroxidation and induce oxidative stress in brain.

An elevation in the content of glutathione has been observed in the astrocytes treated with pathological concentrations of ammonia (Murthy *et al.,* 2000). Glutathione has multiple roles in the functioning of cell and its survival which include maintenance of mitochondrial integrity, protection against free radicals (anti oxidant), oxidative stress, metabolism of xenobiotics and has a role to play in mitochondrial permeability transition (Janaky *et al.,* 1999; Cooper and Kristal, 1997b; Wilson, 1997). The studies of Kosenko and his group (Kosenko *et al.,* 1998; 1997) have indicated that the production of NO and other super

oxides in the brain in hyperammonemic conditions influence the GSH levels.

Collapse of $\Delta\Psi_m$ and permeability change which is a consequence of oxidative stress has been reported in mitochondria of astrocytes exposed to pathological concentrations of ammonia and this was prevented by pretreatment of the cells with methionine sulfoximine (Bai *et al.,* 2001).

The above said results indicated that at least in astrocytes ammonia induces the production of free radicals and affects the mitochondrial function. However, it should be mentioned that most of these studies were carried out on cultured astrocytes. Such studies do help in identifying the cellular site of action and in avoiding the complications arising out of the inter organ and inter cellular interactions in *in vivo* studies, other toxic/ protective factors produced in the body in response to the liver damage. Moreover, *in vivo* metabolic response of neuronal cells is governed by various blood borne factors and the interaction between neurons and glial cells. Such interactions are eliminated in the primary cultures. Even the drugs which protect the cells in cultures need not exert the same action under *in vivo* conditions due to modifications by other tissues or may even prove to be toxic.

Since the mitochondria are the major sites of free radical generation they are closely associated with the oxidative stress. As of today very scanty information is available on the role of cerebral mitochondria in generation of free radicals and imposing oxidative

stress leading to its dysfunctions in conditions of hepatic encephalopathy.

Overall, the present literature suggests that the above conditions prevailing in brain during FHF or hyperammonemic conditions are thought to be highly favorable for the generation of excessive free radicals and induction of oxidative stress. However there is no direct evidence of demonstration of these conditions in brain during FHF and hence this sets the stage for a detailed investigation on this aspect.

Scope of the Present Work

Fulminant hepatic failure is not uncommon and may even assume epidemic proportions. Viral infections and ingestion of hepatotoxins appear to be the primary reasons. A wide range of neurological, neuropsychiatric and neuromuscular changes are associated with the conditions of FHF. Clinical outcome of the patients with FHF depends on the number of surviving hepatocytes, age, sex, etiology and the stage at which the patient is provided with medical help. There is no specific treatment or drugs to be administered to the patients with Fulminant Hepatic Failure and the outcome depends on the supportive intensive care and better patient management. Even then, management of FHF patients is less rewarding.

The Present work is aimed at evaluating the changes in the functioning of cerebral mitochondria leading to the production of free radicals with a concomitant oxidative stress in conditions of hepatic encephalopathy. For this purpose an animal model of Fulminant Hepatic Failure (FHF) was used. Animal model for FHF was generated by using thioacetamide, a well-known selective hepatotoxin. The present study will contribute towards the understanding of the pathophysiology of FHF and also help in developing an unified hypothesis.

Methodology

2.1 Materials

ADP, Alanine, Aspartate, ATP, Bovine serum albumin, Brilliant blue-G250, Citrate, Coenzyme A, Dithiothreotol, 5-5-dithiobis-2-nitrobezoicacid, EDTA, Glutamate, HEPES, Isocitrate dehydrogenase, Lactate dehydrogenase, Malate dehydrogenase, Nicotinamide adenine Dinucleotide, Nicotinamide adenine Dinucleotide phosphate, Nicotinamide adenine Dinucleotide phosphate reduced, Nicotinamide adenine dinucleotide reduced, Oxaloacetate, Phenazine methosulfate, Pyridoxal-5-phosphate, Sodium pyruvate, Thiamine pyrophosphate, Tris, α-ketoglutarate, malate, γ-amino butyric acid, 2,4-idophenyl-3,4-nitrophenyl-5-phenyl tetrazolium chloride, acetyl CoA, oxaloacetate, isocitrate, thiamine pyrophosphate, Dichlorophenol indophenol (DCPIP), Ubiquinone, Cytochrome c, decylubiquinol, Rotenone, Antimycin, Mannitol, SDS, o-pthalaldehyde, were purchased from Sigma chemical Co., USA. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Triton X -100 was procured from Koch-Light chemicals, U.K. Rest of the chemicals which are not mentioned here, were procured from the local companies and were of high quality.

2.2 Animals

Male rats (~300g) of Wistar strain were used in the present study. The animals were kept in cages (4 per cage) at 25 ± 2°C with 12h day-night cycles in the animal house facility available at the

University of Hyderabad. Animals had free access to food (Balanced pellet diet from Hindustan Lever Ltd.,) and water.

2.3 Drug Treatment

Thioacetamide (TAA, 300 mg/kg body weight) dissolved in physiological saline was administered intraperitoneally for 2 days at a 24 h interval. Animals were killed at different time periods after the administration of the second dose. Food and water were provided to the animals ad *libitum.* Control rats received normal saline to serve as vehicle controls. All the rats were given a 25ml/kg body weight of supportive therapy which consisted of 5% dextrose and 0.45% saline with 20 mequiv/L of potassium chloride (Norton *et al.,* 1997).

2.4 Preparation of Serum

Blood from normal and thioacetamide induced rats at specific intervals of time was drawn by cardiac puncture into a syringe and was then transferred into clean dry centrifuge tubes. It was allowed to stand for 30 minutes at room temperature without any disturbance and was allowed to clot. Serum was separated from the clotted blood by centrifugation at 5,000 rpm at 4°C. This was used for the estimation of glucose, urea, proteins and enzymes such as aspartate and alanine amino transferases.

2.5 Preparation of Serum for Ammonia Estimation

Serum was deproteinized by adding an equal volume of 10% perchloric acid (v/v). This was allowed to stand for 15 minutes for complete precipitation of proteins. The tubes were centrifuged at 5,000

rpm for 10 minutes at 4°C. The supernatant was neutralized with saturated potassium carbonate till pH was 7.0. Tubes were kept in ice for 15 minutes and they were then centrifuged at 10,000 rpm for 15 minutes at 2°C to remove precipitated potassium perchlorate. The supernatant was used for the estimation of ammonia.

2.6 Preparation of Brain and Liver Extracts for Ammonia Estimation

Rats were decapitated and the head was allowed to fall into liquid nitrogen and frozen at this temperature for 10-15 minutes. Brains were chiseled out with pre-cooled (with liquid nitrogen) stainless steel chisel and powdered with stainless steel mortar and pestle at the temperature of liquid nitrogen. Liver was excised and plunged into liquid nitrogen. After 10 minutes tissue was powdered and as described above for brain. Powdered tissues were transferred into pre-weighed tubes containing 3 ml of ice-cold 10% perchloric acid and the tubes were weighed again. The powder was dispersed well and homogenized in Potter-Elvehjem glass homogenizer with Teflon pestle. Samples were allowed to stand for 15 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant thus obtained was neutralized as described for the serum and used for the assay of ammonia.

2.7 Preparation of Liver Sample for Biochemical Estimations

Liver was excised from the normal and thioacetamide treated rats and was transferred into a beaker containing ice-cold 0.32 M

sucrose. The tissue was gently pressed between Whatman No. 1 filter papers wetted with sucrose to remove blood present in the tissue. Tissue was cut into small pieces weighed and homogenized in 0.32M sucrose to get required percentage homogenate. This was used for the estimation of enzyme activities and protein content.

2.8 Biochemical Characterization

2.8.1 Estimation Ammonia

Ammonia was estimated in the neutralized PCA extracts of serum, liver and brain by the method of Ratnakumari and Murthy (Ratnakumari and Murthy, 1990). To 1ml of the supernatant 1.5 ml of phenol-nitroprusside reagent (containing 50g of phenol and 250mg sodium nitroprusside in 3.75 liters of water) and 2 ml of sodium hypo chlorite reagent (8.4 g sodium hydroxide, 8.92 g disodium hydrogen orthophosphate and 10ml of 5% sodium hypo chlorite per liter) were added. After 20 minutes at room temperature, the colour intensity was measured at 630 nm against distilled water blank. Ammonium chloride (0.1-1.0 mmoles) was used as a standard.

2.8.2 Estimation of Glucose and Urea

Glucose and Urea levels in serum and in liver extracts were measured by using the diagnostic kits from Glaxo. These kits were meant for the estimation in human samples. Hence it was very much necessary to standardize the same kit for the present study in the rat samples.

Glucose estimation by the kit is based on the glucose oxidase/peroxidase (GOD/POD) method. The principle of this assay is that Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidizes phenol which combines with 4-Aminoantipyrine to produce a red coloured quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration in the sample.

$$\text{D-glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Aminoantipyrine} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinoneimine} + H_2O$$

1ml of the working enzyme reagent was added to the 20 µl sample. It was mixed well and incubated at 37°C for 10 minutes. Absorbance of the sample was measured against a blank (working enzyme) on a double beam Shimadzu-1601-UV-Visible spectrophotometer at 505 nm.

Urea in the serum and liver were measured by Diacetyl monoxime (DAM) method. The principle in this assay is that urea reacts with DAM in an acidic medium to produce a coloured complex. The colour is intensified by using thiosemicarbazide and a cadmium salt. The absorbance of the coloured complex is proportional to the urea concentration in the sample.

To 10 µl of the sample 1ml of urea reagent and 1ml of the DAM reagent were added and mixed well. The final volume in the tubes was made to 6 ml with de-ionized water. The tubes were kept in a water

bath at 100°C for 10 minutes, cooled under tap water and then the absorbance was measured against blank (urea reagent + DAM reagent) at 520 nm.

2.8.3 Determination of Activities of Aminotransferases

2.8.3.1 Aspartate aminotransferase (AST)

Activity of aspartate aminotransferase was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500 µl of 160 mM of potassium phosphate buffer (pH 7.4), 25 µl of 20 mM aspartic acid (pH.7.4), 50 µl of 18 mM α-ketoglutarate (pH.7.4), 25 µl of 0.4 mM NADH, 5 µl malate dehydrogenase (0.5 mg protein/ ml), and 20 µl of sample. The reaction was started by the addition of α-ketoglutarate. Change in the absorbance was measured at 340 nm for 10 minutes at one minute interval. Activity of the enzyme in liver was expressed as |imol/g wet weight. h, while the serum activity was expressed as µmol/ml. h.

2.8.3.2 Alanine Aminotransferase (ALT)

Activity of alanine aminotransferase activity was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500 µl of 160 mM of potassium phosphate buffer (pH 7.4), 25 µl of 40 mM alanine (pH.7.4), 25 µl of 18 mM α-ketoglutarate (pH.7.4), 25 µl of 0.4 mM NADH, 5 µl lactate dehydrogenase (0.5 mg protein/ ml), and 2 µl of sample. The reaction was started by the addition a-ketoglutarate. Change in the absorbance

was measured at 340 nm for 10 minutes at one minute interval. Activity of the enzyme in liver was expressed as mentioned above for AST activity.

2.8.4 Determination of Protein Content

Protein content in an aliquot of brain and liver preparations was estimated by the method of Lowry *et al.,* (1951). Protein content in the serum was estimated using biuret reagent by the method described by (Varley, 1969). For the mitochondrial studies protein content was determined by the method of Bradford (1976). Bovine serum albumin was used as standard.

2.9 Preparation of Plasma

20µl of heparin was added to clean and dry test tubes. Rats were decapitated and approximately 2 ml of blood was collected into these tubes and mixed thoroughly. This was centrifuged at 5000 rpm for 10 min. The upper clear pale straw coloured supernatant, plasma, was aspirated carefully.

2.10 Determination of Prothrombin Time

Prothrombin time was determined by using the diagnostic kit, Liquiplastin, supplied by Tulip Diagnostic (P) Ltd. Liquiplastin is a ready to use calcium- thromboplastin liquid reagent .

In a tube 0.1 ml of the plasma was taken and placed in water bath at 37°C for 5 min. To this tube, 0.2 ml of Liquiplastin reagent (prewarmed at 37°C) was added, mixed the contents and

simultaneously started a stop watch. The time required for the appearance of the first fibrin strand was recorded.

2.11 Histopathology

Liver histology was studied in control and in thioacetamide treated rats at different time periods (6, 12, 18, and 24 h) after the administration of drug. Animals were anaesthetized with ether and the portal vein was cannulated with Viggo Venflon-2 I .V. cannula with an injection valve and PTFE catheter (0.8mm O.D; 22G). Leur-lock end of the catheter was connected to 0.9% (w/v) ice-cold saline reservoir while the injection valve was connected to 10% buffered formalin (pH 7.4) through a two way Teflon valve. Both saline and formalin were allowed to flow under gravitational force (35-40ml/min). Initially liver was perfused with 0.9% saline till the colour of the tissue turns to pale brown (5-8 min of perfusion). At this juncture the two way valve was opened in such a way that formaldehyde reservoir was connected and saline reservoir was disconnected. Perfusion with formalin was continued for 10 min to achieve total fixation of the tissue. Liver tissue was then excised, cut into 1mm cubes and stored in Bouin's fluid for 3-4 days. The tissue was dehydrated by passing through graded series of ethyl alcohol (30 min each in 30% and 50% alcohol; 80 min in 70% alcohol; 30 min each in 90%, 95% and 100% alcohols).

Then the tissue was transferred to 1:1 (v/v) Acetone : Alcohol mixture for 10 min and then into acetone for 5 min followed by transferring the tissue into acetone: benzene mixture(1:1; v/v) for one

hour; to Benzene for 1 h and then to benzene: paraffin wax (1:1; v/v) for 2 h. The last step was done in an oven at 55°C and was changed after every one hour for three times. Finally, the tissue was transferred to molten wax in an oven maintained at 55° C. The tissue was then embedded in paraffin wax with in 24 h.

2.11.1 Preparation of Wax

Wax (melting point 60-62°C) was seasoned by melting (60-62°C) and cooling for at least 4-5 times. To 100 g of seasoned wax, 1 g of bee wax was added and allowed to melt. This was thoroughly mixed and stored in frozen condition. Requisite quantity of this wax was melted just before use.

2.11.2 Preparation of Tissue Sections

Sections (5-7μm) were cut with a rotary microtome. Ribbons were placed on slides pre-coated with Meyer's albumin and spread by slight warming of the slides.

2.11.3 Staining of Sections

Slides with tissue sections were placed successively in xylene (twice-5 min each), absolute alcohol (5 min), 90% alcohol (5 min), 70%alchohol (5 min), 50% alcohol (5 min), 30% alcohol (5 min), distilled water (10-15 min), 3% iron alum (30 min), tap water (10 min), distilled water (2 min) haematoxylene (1 hour), 1% iron alum (allowed to differentiate), running tap water (30 min), distilled water(5 min), 30% alcohol (5min), 50% alcohol (5 min), 70% alcohol (5 min), alcoholic eosin (2 min), 90% alcohol (2-3 min), absolute alcohol (2-3 min),1:1

alcohol and acetone (5 min), acetone (5 min), acetone and xylene (5 min), xylene (5 min), and finally mounted in DPX. The slides were observed under Nikon Labphot microscope and photographs of the randomly selected areas were taken by an observer not aware of the treatments.

2.12 Mitochondrial Isolation by Sucrose Density Gradient

Brain mitochondria were isolated from adult Wistar rats. Following decapitation, brains were rapidly dissected out and placed in ice-cold isolation buffer consisting of 225 mM Mannitol, 2 mM EDTA and 5 mM Hepes (pH 7.40). Brains were rinsed in the above buffer and were blotted between two filter papers to remove coagulated blood and capillaries. Brain was weighed and homogenized in the isolation buffer using Potter-Elvejhem homogenizer to obtain a 10% (w/v) homogenate.

The homogenate was centrifuged at 1,200g for 10 minutes. The pellet (P_1) was discarded and the resulting supernatant was centrifuged at 10,000g for 10 minutes. The pellet (P_2) obtained was washed with the same buffer, and centrifuged at 15,000g for 15 minutes. The final pellet (P_3) was suspended in 2 ml 0.32 M sucrose.

A discontinuous density gradient of sucrose was prepared using 1 M, 0.8 M, 0.32 M sucrose solutions. The P_3 pellet, suspended in 0.32M sucrose, was loaded on the top of the gradient. This tube was then centrifuged at 65,000g for 75 min. The mitochondria were obtained as a pellet in the 1M layer. This was suspended in 0.32 M

sucrose and the protein content was adjusted to 1 mg/ml. The mitochondria isolated by this method was used in the first part of the studies (to study the TCA cycle enzyme activities) where the coupling of the mitochondria was not an important factor. For the rest of the studies mitochondria was isolated by using a Ficoll density gradient.

2.13 Mitochondrial Isolation by Ficoll- 400 Density Gradient

Mitochondria were also isolated from the cerebral cortex of rats according to the method of Cotman (1974) as described by Ratnakumari and Murthy (1990). Following decapitation, brains were rapidly dissected and placed in ice-cold saline. The tissue was blotted between two filter papers to remove coagulated blood and capillaries. Tissue was weighed and homogenized in 0.32 M sucrose using Potter-Elvehjem homogenizer to obtain a 10% (w/v) homogenate. The homogenate was centrifuged at 720g for 5 minutes to obtain a pellet (P_1) consisting of unbroken cells, debris, nuclei and capillaries. The supernatant (S_1) was centrifuged at 15,000g for 12 minutes to obtain a pellet (P2) consisting of mitochondria, synaptosomes and myelin. The pellet (P_2) was resuspended in 5 ml of 0.32 M sucrose and was layered on top of a preformed discontinuous density gradient (consisting of 10ml each of 4%, 6%, and 13% Ficoll-400 in 0.32 M sucrose) and centrifuged at 63,500g for 45 minutes. This resulted in the separation of myelin in 4% Ficoll layer, synaptosomes (at the interphase of 6%-13%ficoll) and mitochondria (pellet below the 13% ficoll layer). Myelin and synaptosomes were aspirated out with a pasture pipette and discarded. The mitochondrial pellet was resuspended in

0.32M sucrose and centrifuged at 20,000g and the final pellet was used for various assays.

2.14 Assessment of Purity of the Isolated Subcellular Fractions

In order to avoid the contamination of the mitochondria with the synaptosomes and with the vesicles formed by the sheared nerve endings during the homogenization, metabolically active contamination-free nonsynaptic mitochondria from the cortex was isolated. The purity of the fractions was assessed by estimating activity of marker enzymes - Succinate dehydrogenase for mitochondria and glutamic acid decarboxylase for synaptosomes.

2.14.1 Succinate Dehydrogenase

Activity of SDH was determined by the method of Nandakumar *et al.,* (1973). The assay mixture (1ml) containing 40 mM succinate, 100 mM phosphate buffer pH (8.0), 4 mM INT, 1.6 µM PMS, 50 µl of sample was incubated for 20 minutes at 37°C . The reaction was stopped by adding 2 ml of glacial acetic acid. The colour was extracted into 5 ml of toluene and absorbance was read at 500 nm. Formazan standards were prepared by reducing various amounts of INT with ascorbic acid in alkaline medium. These were used for calculating enzyme activity.

2.14.2 Glutamic Acid Decarboxylase (GAD)

The reaction mixture (250µl) containing 100 µl substrate mixture (3.6 mg glutamic acid in 25 µl of 1N NaOH, 7.8 mg NaH_2PO_4 in 1 ml

distilled water), 5 |il of pyridoxal 5 phosphate (2.5 mg in 10 ml), 100 µl of sample was incubated at 37°C for 20 minutes. The reaction was stopped by boiling for 10 minutes. The mixture was then centrifuged and 20 µl of supernatant was spotted on to a Whatman filter paper 1 and developed in butanol : acetic acid: water (65:15:25). The chromatogram was sprayed with ninhydrin. GABA spot was eluted with 75% alcohol containing 0.005% $CuSO_4$. The colour was read at 515nm and compared with values obtained using GABA standard.

2.15 Assay of Citric Acid Cycle Enzymes

2.15.1 Pyruvate Dehydrogenase (PDH)

The method described by Hinman and Blass (1981) was used for PDH

$$\text{Pyruvate} + \text{CoA} + \text{NAD} \longrightarrow \text{Acetyl CoA} + \text{CO} + \text{NADH} + H^+$$

$$\text{NADH} + H^+ + \text{PMS} + \text{INT} \longrightarrow \text{Formazan} + NAD^+ + \text{PMS}$$

The reaction mixture consists of 50mM potassium phosphate buffer (pH. 7.8), 2.5 mM NAD^+, 0.2 mM thiamine pyrophosphate, 100 µM CoA, 0.3 mM dithiothreotol, 5 mM pyruvate, 1mM magnesium chloride, 6.5 µM Phenazine methosulfate (PMS; intermediary electron acceptor), 300 µM 2,4-idophenyl-3,4-nitrophenyl-5-phenyl tetrazolium chloride (INT; terminal electron acceptor), 5 mM pyruvate, 0.2% Triton X -100 and 20 µg of mitochondria. After pre-incubation for 5 min at 37°C, CoA was added and the change in absorbance was read at 500 nm at 15 second intervals for 5 min. Activity was expressed as nmoles/min.mg protein.

2.15.2 Citrate Synthase

The method of Shepherd and Garland (1969) was adopted for the assay of citrate synthase.

Acetyl CoA + Oxaloacetate ——— • Citrate + CoA (SH)

Free thiol groups of CoA formed in the course of the reaction were reacted with 5,5'dithio-bis(2-nitrobenzoic acid) (DTNB) and the rate of formation of DTNB-thiol complex was measured at 412nm.

Free thiol groups + DTNB ———▶ DTNB-thiol coloured complex

Assay mixture consisted of 96 mM Tris-HCl buffer (pH. 8.0), 97.2 μM DTNB, 0.24 mM oxaloacetate, 48 μM acetyl CoA, 0.2% Triton X -100 and 20 μg of mitochondrial protein. Reaction was initiated by the addition of oxaloacetate. Increase in the absorbance at 412 nm was recorded at 5 sec interval for 5 minutes. Enzyme activity was calculated using the molar extinction coefficient of DTNB-thiol complex (1.36×10^6). Activity was expressed as nmoles/min.mg protein.

2.15.3 Isocitrate Dehydrogenase (ICDH) (NAD)

This enzyme was assayed by the method of Plaut (1969)

Isocitrate + NAD^+ ———▶ 2-oxoglutarate + CO_2 + NADH + H^+

NADH + H^+ + PMS + INT ———▶ Formazan + NAD^+ + PMS

The reaction mixture contained 33.3 mM Tris-acetate buffer (pH. 7.2), 1 mM magnesium chloride, 6.7 mM ADP, 333 μM NAD^+, 5.28 mM isocitrate, 6.52 μM PMS, 300 μM INT, 0.2% Triton X -100 and 20 μg of

mitochondrial protein. The reaction was initiated by the addition of isocitrate and the change in absorbance was followed at 500 nm. Activity was expressed as nmoles/min.mg protein.

2.15.4 2-Oxoglutarate Dehydrogenase (2-OGDH)

The method of Reed and Mukherjee (1969) was used for the assay of this enzyme.

2-oxoglutarate + CoA + NAD^+ ——• Succinyl CoA+ CO_2 + NADH+ H^+

NADH + H^++ PMS + INT ——▶ Formazan + NAD^+ + PMS

The reaction mixture consists of 50 mM potassium phosphate buffer (pH. 8.0),1 mM magnesium chloride, 2 mM NAD^+, 0.2 µM thiamine pyrophosphate, 60 µM coenzyme A, 1 mM 2-oxoglutarate, 6.52 µM PMS, 0.3 mM INT, 0.2% Triton X -100 and 20 µg of mitochondrial protein. The reaction was initiated by the addition of CoA and the change in absorbance was followed at 500 nm. Activity was expressed as nmoles/min.mg protein.

2.15.5 Succinate Dehydrogenase (SDH)

Succinate dehydrogenase was assayed as per the method of Veeger *et al.,* (1969)

Succinate + FAD ——• Fumarate + $FADH_2$

$FADH_2$ + PMS + DCPIP (oxidized; chromobase) ——•

FAD+ PMS+ DCPIP (reduced; leucobase)

The assay mixture consists of 50 mM potassium phosphate buffer (pH. 7.6), 40 mM succinate, 6.52 μM PMS, and 50 μM dichlorophenol indophenol (DCPIP), 0.2% Triton X -100 and 20 μg of mitochondrial protein. Reaction was initiated by the addition of succinate and the reduction of DCPIP was followed at 600 nm for 5 min at 15 sec intervals. Activity was expressed as nmoles/min.mg protein.

2.15.6 Malate Dehydrogenase (MDH)

Activity of this enzyme was studied by the method of Yoshida (1969).

$$\text{L-Malate} + NAD^+ \longrightarrow \text{Oxaloacetate} + NADH + H^+$$

The reaction mixture in the direction of oxaloacetate formation, consisted of 83.2 mM Tris-HCl buffer (pH. 8.8), 3.2 mM malate, 0.33 mM NAD^+, 6.52 μM PMS, 0.3 mM INT, 0.2% Triton X -100 and 2 μg of mitochondrial protein. Reaction was initiated by the addition of malate and was followed at 500 nm. Activity was calculated by using the extinction coefficient (ε_{mM}=6. 22) of NADH. Activity was expressed as nmoles/min.mg protein.

2.16 Electron Transport Chain Enzyme Activities

2.16.1 NADH - Ubiquinone Oxidoreductase (Complex I)

Mitochondrial respiratory complex I activity was measured according to the method of Ragan *et al.,* (1987) The decrease in the absorbance due the oxidation of NADH at 340 nm leading to the reduction of ubiquinone (C0Q1) to ubiquinol was measured. The

reaction mixture contained 20 mM phosphate buffer, pH 7.2, 10 mM $MgCl_2$, 0.15 mM NADH, 1 mM KCN, 2.5 mg BSA (fatty acid free) and the mitochondrial sample. The reaction was initiated by the addition of 50 µM C0Q1 and it was run at a temperature of 30°C. After measuring the activity for 5 minutes 10 µM rotenone was added and the activity was further measured for another 5 min. The complex I activity measured was rotenone sensitive NADH-ubiquinone oxidoreductase. Activity was expressed as nmoles /min. mg protein.

2.16.2 Succinate - Ubiquinone Oxidoreductase (Complex II)

The activity of complex II was determined as per the method of Hatefi and Stiggal (1978) as described by Cardoso *et al.,* (1999). The disappearance of the colour of the DCPIP dye due to the secondary reduction of the dye by the ubiquinol that is formed as a result of the reduction of the ubiquinone (C0Q2) compound was measured at 600nm. The final reaction mixture contained 50 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 1 mM KCN, 75 µM DCPIP dye, 0.1 mM EDTA (di-potassium salt), 10 µM rotenone and mitochondrial sample. The reaction was initiated by the addition of 50 µM CoQ_2. The activity was expressed as nmoles/min. mg protein.

2.16.3 Ubiquinone-Cytochrome-c Oxidoreductase (Complex III)

Mitochondrial complex III activity was measured according to the method of Birch-Machin *et al.,* (1994). This enzyme donates the electrons from ubiquinol to cytochrome c, thus resulting in the reduction

of cytochrome c. This reduction of the cytochrome c was measured at 550nm with a reference wave length of 580 nm. The final reaction mixture contained 35 mM phosphate buffer, pH 7.5, 5 mM $MgCl_2$, 2.5 mg BSA (fatty acid free), 60 µM decylubiquinol, 1.8 mM KCN, 50 µM rotenone and mitochondrial sample. The reaction was initiated by the addition of 125 µM cytochrome *c.* the reaction was run for 3 min at 30°C and then 3 µg/ml antimycin A was added and the reaction was run for another 3 min. The activity of complex III was found to be antimycin sensitive. The activity of the enzyme was expressed as nmoles/min. mg protein.

2.16.4 Cytochrome-c Oxidase (Complex IV)

The activity of complex IV was measured according to the method of Wharton and Tzagoloff (1967). The activity was determined by monitoring the decrease in the absorbance at 550 nm due to the oxidation of cytochrome c. Before the reaction was done, cytochrome c was reduced by the addition of a pinch of ascorbate. The mixture was then dialyzed for 24 h against 0.01 M phosphate buffer, pH 7.0, in a cold room maintained at 4°C. This was used for the assay of the activity of this enzyme. The reaction mixture contained 0.01 M phosphate buffer, pH 7.0, 5 µM reduced cytochrome c. The reaction was initiated by the addition of the mitochondrial sample and was run at 30°C. The activity was expressed as nmoles /min. mg protein.

2.17 Measurement of Mitochondrial Respiration

Oxygen consumption in the nonsynaptic mitochondria was measured by using a Clark oxygen electrode (Gilson Model 5/6 Oxygraph). For this purpose mitochondria isolated by the Ficoll density gradient was suspended in a medium containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Respiratory measurements were carried out in the medium consisting of 25 mM sucrose, 75 mM Mannitol, 5 mM KH_2PO_4 100 mM KCl, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4, in the presence of the respiratory substrate containing 0.5 M succinate or 5 mM pyruvate and 2.5mM malate. 200 nmoles of ADP/mg protein was added in order to measure the state 3 respiration and the P/O ratio. State 3 and state 4 refer to the active and resting states of the mitochondrial respiration respectively. The respiratory control ratio was calculated as the ratio of state 3 to that of state 4 respiration. The P/O ratio was calculated as the amount of ADP to that of the oxygen consumed during the state 3 respiration. The respiration measurements were performed at 27°C.

2.18 Measurement of Mitochondrial Swelling

Mitochondrial swelling was measured according to the method of Packer (1967). About 40 |ig of the mitochondrial protein was suspended in a buffer containing 10 m mol Tris, 50 m mol sucrose, 5m mol $MgCl_2$, 10 m mol KCl and 0.25 m mol ADP in 1 ml reaction mixture. The reaction was started by the addition of succinate (5 m mol/ lit).

Swelling was measured by following the change in absorbance at 540 nm in a Shimadzu-1601 UV-Visible spectrophotometer.

2.19 Preparation of Tissue for Transmission Electron Microscopy

The cerebral cortex tissue blocks were cut into 400 μm thick sections with a vibratome. Slices were washed in cold 0.1 M sodium cocodylate buffer and kept in 2.5% glutaraldehyde in 0.1 M cocodylate buffer until further processing. When processing resumed, slices were washed in cold cocodylate buffer and were then post fixed in 1% osmium tetraoxide in cold cocodylate buffer for 1 hour. After osmium tetroxide step, 400 μm thick sections were washed in 0.1% cocodylate buffer. 2x2 mm sections were cut out from the tissue slices, dehydrated in a graded series of ethanol and embedded in Spur Epon. Blocks were trimmed and semi thin 0.5 μm hick sections were cut out with an ultra microtome stained with toulidine blue and examined by light microscope for an overall view. Ultra thin 70-90 nm thick sections were then cut, picked up on 200 mesh copper grids, double stained with uranyl acetate and lead citrate and scanned in Joel 100CX electron microscope.

2.20 Oxidative Stress

In order to evaluate the involvement of oxidative stress in the nonsynaptic mitochondria isolated from the cerebral cortex of rat brain in conditions of thioacetamide induced FHF, lipid peroxidation, total thiols, various antioxidant enzymes have been evaluated.

2.20.1 Lipid Peroxidation

Malondialdehyde, a by product of lipid peroxidation, was determined by the classical thiobarbiturate assay of Ohkawa *et al.,* (1979) as described by Kosenko *et al.,* (2003). In brief, the brain homogenates were prepared in 1.15% KCl. Mitochondria were prepared as described earlier and later suspended in 1.15% KCl. To 0.1 ml of the mitochondrial sample, 0.2 ml of 8.1% SDS, 1.5 ml of acetic acid (20%, pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid were added, and the final volume was made up to 4ml. This total mixture was incubated at 90°C for a period of one hour. The samples were then cooled and centrifuged at 1000g for 10 min at room temperature. The absorbance of the supernatant was measured at 535 nm with malondialdehyde as the standard.

2.20.2 Assay of Glutathione Peroxidase (GPx)

Activity of Glutathione peroxidase (GPx) (EC 1.11.1.9) was measured according to the method described by Lawrence and Burk (1976)

One unit of activity was defined as one nmole of NADPH oxidized per min.

$$ROOH + 2\ GSH \longrightarrow ROH + H_2O + GSSG$$

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance per min X Volume of the reaction mixture (ml)}}{\text{e NADPH (6.22) X volume of the enzyme (ml).}}$$

Specific activity was expressed as units per mg protein, where one unit is defined as one nmole of NADPH oxidized per minute.

2.20.3 Glutathione Reductase Assay (GR)

Activity of glutathione reductase (GR) (EC 1.6.4.2) was determined by following the procedure of Carlberg and Mannervik (1975). In brief, the reaction mixture (final volume 1ml) consists of 0.2M sodium phosphate buffer pH 7.0, 0.2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was initiated by the addition of mitochondrial protein sample and the oxidation of NADPH was recorded as decrease in absorbance at 340 nm for five minutes. Nonspecific oxidation of NADPH was measured in the absence of added GSSG and the enzyme activity was calculated using the molar extinction coefficient of NADPH (6.22 mM^{-1} cm^{-1}). Specific activity was expressed as units per mg protein, where one unit was defined as one nmole of NADPH oxidized per minute.

$$NADPH + H^{+} + G\text{-}S\text{-}S\text{-}G \longrightarrow NADP^{+} + 2GSH$$

Activity was calculated according to the following equation.

$$\frac{\text{Difference in absorbance per min X Volume of the reaction mixture (ml)}}{\text{E NADPH (6.22) X volume of the enzyme (ml).}}$$

2.20.4 Total Thiols

Total thiols were estimated as per the method of Sedlak and Raymond (1968). Aliquots of 0.1ml sample were mixed with 1.5 ml of 0.2 M Tris buffer, pH 8.2 and 0.1ml of 0.01M DTNB. The mixture was

made up to 10ml with 7.9 ml of absolute methanol and it was incubated for 30 minutes. The mixture was then centrifuged at 3000 rpm for 15minutes and the absorbance of the supernatant was read at 412 nm. The molar extinction coefficient of 13100 was used to calculate total thiols.

2.20.5 Assay of Superoxide Dismutase (SOD)

Total SOD activity was assessed according to the method of described by Beauchamp and Fridovich (1971) by measuring the degree of inhibition of the reduction of NBT in the presence of Xanthine-xanthine oxidase system. Mn-SOD activity was calculated as the difference between the total activity (Cu,Zn-SOD and Mn-SOD) and the activity measured in the presence of Cu,Zn-SOD inhibitor cyanide. One unit of activity was defined as the amount of enzyme required to inhibit 50% NBT reduction rate.

2.20.6 Preparation of the Sample for Estimation of GSH and GSSG

For the estimation of glutathione, isolated mitochondria were homogenized in potassium phosphate buffer (100 mM, pH 7.4) containing 1mM EDTA and 1ml of 25% orthophosphoric acid for precipitation. The above homogenized mixture was centrifuged at 10,000g for 30 min at 4°C. The supernatant that was obtained after the spin was used for the estimation of GSH and GSSG.

2.20.6.1 Estimation of Glutathione (GSH)

GSH was determined by following the modified method of Hissin and Hilf (1976). To 0.5 ml of the above obtained supernatant, 2 ml of

phosphate-EDTA buffer was added. The final reaction mixture of 2ml contained 20 µl of the mitochondrial supernatant, 1.8 ml of the phosphate-EDTA buffer, and 100µl of o-pthalaldehyde (OPT) solution containing 100 µg of OPT. The contents were mixed thoroughly and incubated for 15 minutes. Fluorescence at 420nm was measured with an excitation at 350 nm.

2.20.6.2 Estimation of GSSG

To 0.5 ml of the supernatant obtained above after spinning at 10,000g for 30 min at 4°C, 200 µl of 0.04M N-ethylmaleimide (NEM) was added and incubated at room temperature for 30 minutes. To this mixture 1.8 ml of 0.1 N NaOH was added. The final reaction mixture of 2ml contained 20 µl of the mitochondrial supernatant, 1.8 ml of NaOH, and 100 (il of o-pthalaldehyde (OPT) solution containing 100 µg of OPT. The contents were mixed thoroughly and incubated for 15 minutes. Fluorescence at 420nm was measured with an excitation at 350 nm.

2.21 Statistical Analysis

The data in this thesis are reported as Mean ± SD of at least five sets of experiments. One way ANOVA was performed to evaluate the statistical differences between the groups. Comparisons between multiple groups were carried out using Newman-Keul's multiple range test (Keul, 1952). $P < 0.05$ was considered statistically significant.

Results & Discussion

Animal Model Development & Characterization

3.1 Development of an Animal Model

Fulminant hepatic failure (FHF) is a clinical syndrome with a rapid onset of severe inflammatory and necrotic liver damage. Neurological disturbances under these conditions range from altered mental status to coma and finally death. These changes occur within hours or days. Frequently death results from brain herniation caused by increased intracranial pressure resulting from brain edema (Blei, 1992).

In order to have a clear understanding of the neurological dysfunctions in fulminant hepatic failure, it is highly desirable to work with human samples. However, availability of human samples would be problematic due to the ethical considerations and it would put a serious restriction in carrying out temporal studies to follow the course of the disease. Even if the material is available, it would be mostly post mortem. Various degrees of post mortal changes might have occurred in the sample due to the time lapse between the death of the patient and collection of the material, and mode of preservation. Drugs administered in the course of treatment would also influence the biochemical parameters in the sample. Hence, it would be difficult to infer whether the results obtained are really the experimental changes or due to the artifacts. These problems can be overcome by developing a suitable animal model that closely mimics the conditions observed in humans.

3.2 Features of an Ideal Animal Model

The following criteria have been proposed by Terblanche and Hickman (1991) in developing an animal model for acute liver failure

1. Evidence of liver failure
2. Death from liver failure: The course of events after insult should reflect human clinical pattern and death should be a direct result of the insult to the liver.
3. Reproducibility: reproducible end points are required to standardize any successful animal model.
4. Reversibility: Animal model developed should be such that FHF must be reversed if suitable treatment is introduced and the animal should survive. This would help to assess new therapeutics.
5. Therapeutic window: Time should be available between insult and death such that the treatment can be initiated and assessed for its effect.
6. Large animal model: Most artificial liver support systems require large animal models such that blood and tissue analysis can take place serially as treatment is being assessed. This is more relevant to humans and makes the scale-up for use in man, less problematic.
7. Minimal hazard to personnel

3.3 Types of Animal Models Available for FHF

The study of animal models for FHF has followed two different approaches.

1. Surgical procedures.
2. Pharmacological procedures

3.3.1 Animal Models Based on Surgical Procedures

Model	Animal	Results	Reference
Partial hepatectomy	Rat	Decreased survival, Increased AST, Late hypoglycemia.	Panis *et al.*, 1997
Total hepatectomy	Pig	Survival 15-26 hours, Preterminal encephalopathy, Hypoglycemia and AST rise	Hickman *et al.*, 1974
Resection/ Ligation model	Rat	Late hypoglycemia, increased ammonia, Lactate and Prothrombin time, encephalopathy III	Eguchi *et al.*, 1997.

These models suffer from the drawback that they are often non-reversible and lack clinical patterns seen in man.

3.3.2 Animal Models Based on Pharmacological Procedures

Model	Animal	Results	Reference
D- Galactosamine	Rats Rabbit Dogs	Encephalopathy, Increased AST, ammonia, hepatic necrosis.	Kepler *et al.*, 1968 Blitzer *et al.*, 1978. Diaz Buxo *et al* ., 1997.
Acetaminophen (Paracetamol)	Dogs Pigs	Encephalopathy and coma.	Kelly *et al.*, 1992. Miller *et al* ., 1976.
Carbon tetrachloride	Rats	Late stage coma	Shi *et al.*, 1998.
Thioacetamide.	Rats	Encephalopathy, Increased AST, ammonia, Prothrombin time, hepatic necrosis, metabolic acidosis.	Bruck *et al.*, 1998, Zimmerman *et al.*, 1989.

The different pharmacological agents sited above have different modes of action for the induction of FHF. The potency and the reproducibility vary with different animals.

3.4 Thioacetamide-Toxicity

Thioacetamide, a selective hepatotoxin, is well known to induce hepatic failure (Albrecht *et al.,* 1990). Within a short period of time after the administration of the drug, thioacetamide is rapidly metabolized to acetamide and thioacetamide-S-oxide by the mixed function oxidases in the body (Cheli and Malvadi, 1984). Acetamide does not have liver necrotizing properties while thioacetamide-S-oxide is further metabolized by cytochrome P-450 monoxygenases to a sulfene, thioacetamide S-dioxide. This thioacetamide S-dioxide is a very highly reactive compound (Hunter *et al.,* 1977, Porter and Neal, 1978). Its binding to the tissue macromolecules might induce hepatic necrosis (Porter and Neal, 1978).

The reason for selecting rat as an animal model is that these animals can easily be bred and handled in the laboratory and also there is enough availability of samples for the biological and morphological characterization at various time points in the disease conditions. Hence, an animal model with fulminant hepatic failure is generated by administering thioacetamide intraperitoneally.

The feeding of the animal decreased considerably after the first dose of TAA and is stopped completely during 12-24 h after its second dose. Moreover the animal became progressively inactive and sleepy

from 6 h after the administration of the second dose. From 12 h after the second dose, these animals showed wobbly gait and loss of Tightening reflexes. This change in the gait and loss of reflex was enhanced after 18 h after the second dose of thioacetamide.

The characterization of the model is done by certain relevant biochemical parameters such as ammonia, urea, glucose and protein levels in the serum. Liver function was also assessed by measuring the activities of aspartate and alanine amino transferases in the serum. Concurrent studies were also carried out in the liver and brain samples.

3.5 Liver Function Tests

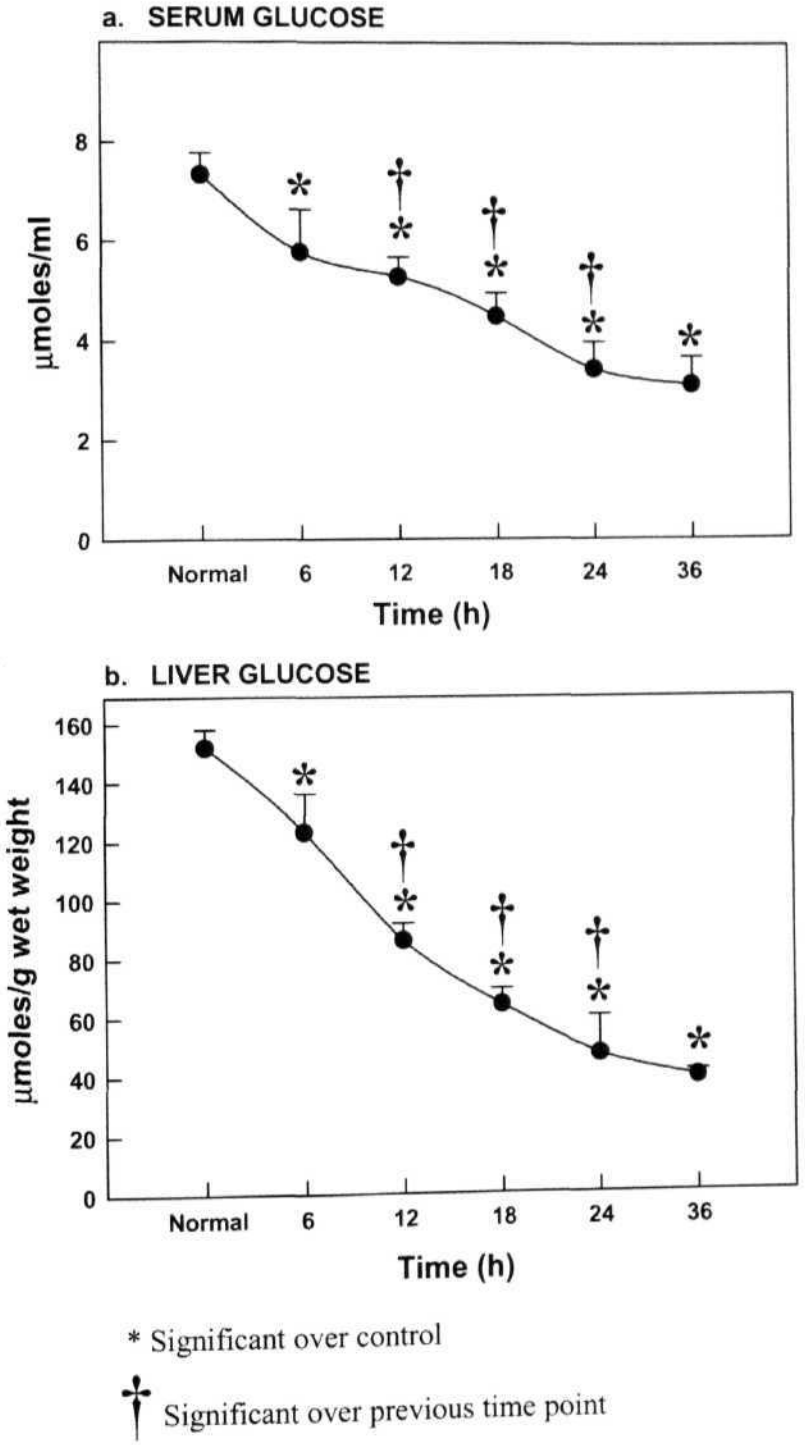
3.5.1 Glucose

Liver is a vital organ that plays a key role in the homeostasis of blood composition, specifically blood glucose levels. So any damage to the liver will be reflected directly in the blood glucose levels. Hence, to assess the liver damage in thioacetamide toxicity, glucose levels were estimated in serum as well as in the liver of normal and drug administered rats at different time intervals. In the present study, serum glucose levels were determined instead of whole blood glucose levels. This was done keeping in mind the fact that blood has different types of cells which also trap glucose. This glucose is used exclusively by these blood cells for their metabolic demands and is not available for other tissues/cells whereas the glucose present in the serum is available not only to blood cells but also to cells in other organs.

The glucose levels were decreased in both serum (Fig. 1a) and liver (Fig. 1b) after the administration of thioacetamide. In liver the glucose level was decreased by 50 % of the control value within 12 h after administration of thioacetamide and by 36 h the glucose level in liver was decreased by 75%.

The fall in the serum glucose level was not so rapid as compared to that of liver. The magnitude of decrease in serum glucose levels was more or less same (-20%) as that of liver in the initial stages of toxicity. But by 12 h, the decrease in liver glucose level was much higher than those in the serum. At the end of 36 h, serum glucose levels were only half while that of liver was one fourth of the respective control values. The decrease in serum and liver glucose levels might be due to two reasons - liver damage caused by thioacetamide and decreased feeding of the animal. As the animal stops feeding, naturally blood glucose levels would decrease rapidly. Under such conditions, in normal animals, liver would maintain blood glucose levels initially by glycogenolysis and later by gluconeogenesis. However, in the drug treated animals, this process may not be operative in an effective manner due to the hepatotoxic effects of thioacetamide (Wagle *et al.,* 1976; Hoyumpa and Schenker 1985).

Fig. 1: Levels of glucose in the (a) serum and (b) liver of normal and thioacetamide treated rats at various time points



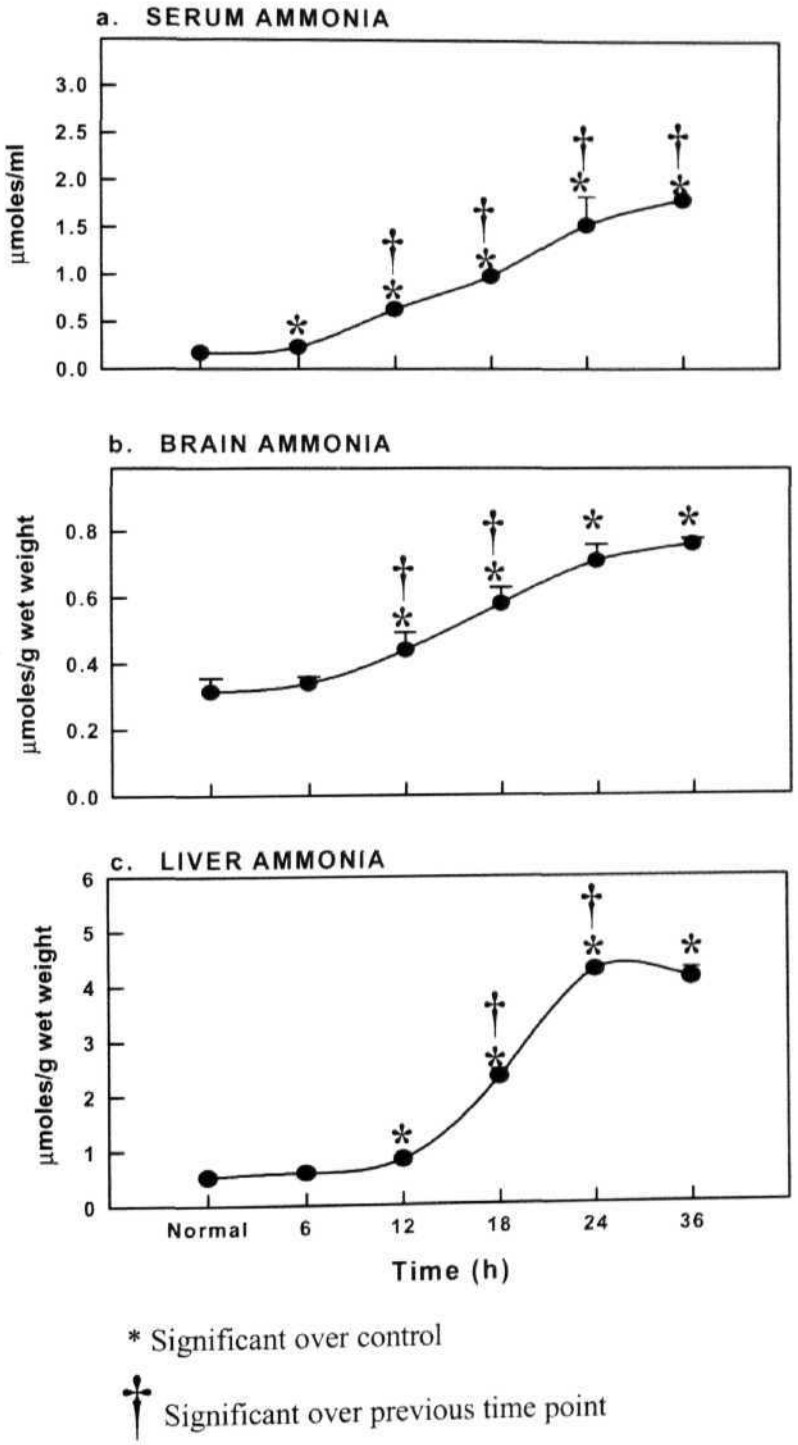
3.5.2 Ammonia

Increase in blood ammonia levels is one of the hallmarks of the liver disease (Norenberg *et al.,* 1996; Albrecht *et al.,* 1998). Hence, ammonia levels in the serum were measured at different time periods after the administration of second dose of thioacetamide. The reason for measuring ammonia levels in the serum but not in the whole blood is same as that given beore.

The ammonia levels in serum (Fig. 2a), brain (Fig. 2b) and liver (Fig. 2c) were increased. Ammonia levels in the serum increased by 4 folds by 12 h after the administration of thioacetamide (second dose) and this increase was progressive. By the end of 36 h the serum ammonia levels were elevated by more than 10 fold when compared to controls. Similar results indicating an increase in the blood ammonia levels were reported by Bruck and his group in Wistar rats. (Bruck *et al.,* 1999;2002).

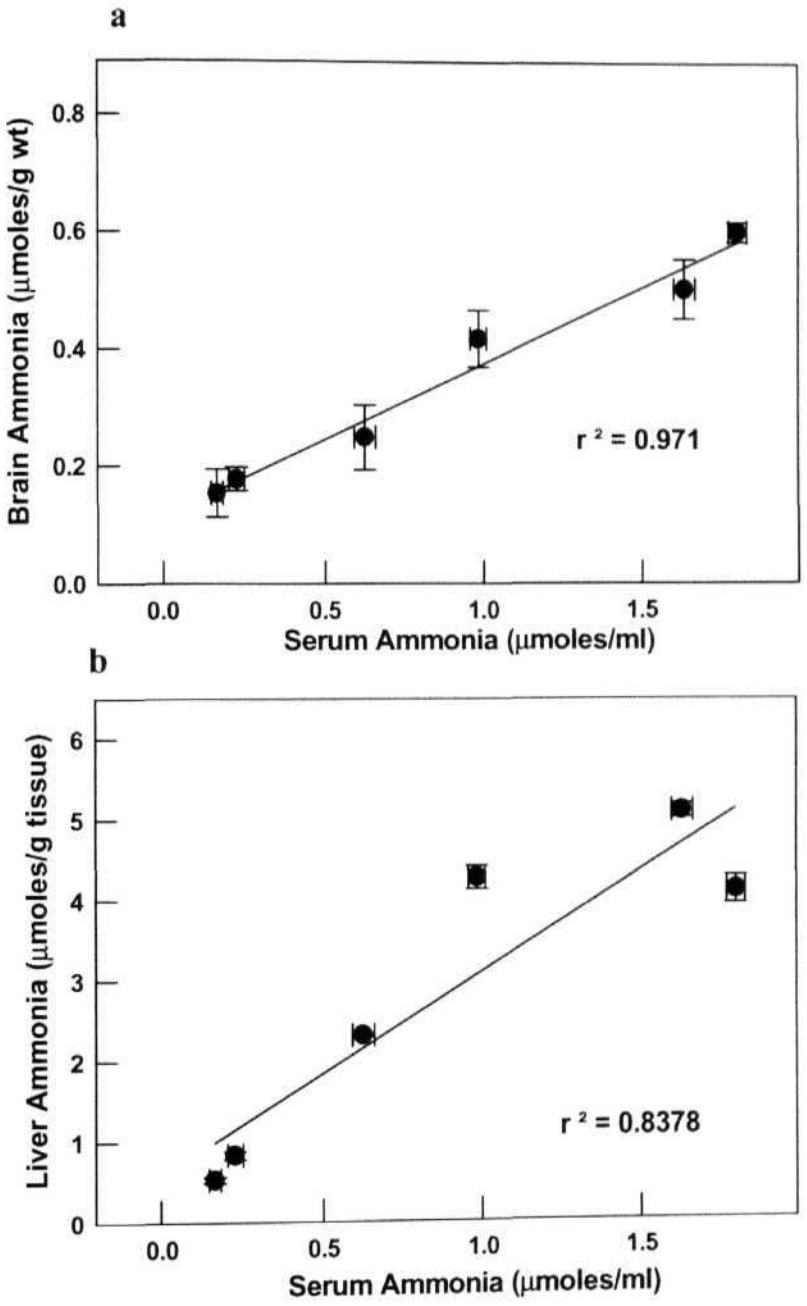
The increased ammonia levels in the serum (Fig. 2a) in the drug treated animals might be due to the increased production or decreased utilization /detoxification of ammonia in the body. Ammonia is produced in the body due to the degradation and metabolism of nitrogenous compounds. The major source of ammonia is by the action of microbes on the dietary nitrogenous compounds, which occurs in intestine. However, it must be mentioned that this may not be the major source for ammonia in the drug treated animals as these animals stop feeding.

Fig. 2: Levels of ammonia in the (a) serum, (b) brain and (c) liver of normal and thioacetamide treated rats at different time periods



Hence, the ammonia should have been produced from internal sources. One such source is liver where amino acids are utilized for the production of glucose through the pathway of gluconeogenesis resulting in the production of ammonia. In addition, intestinal smooth muscle is known to preferentially use glutamine as the source for its energy and this also results in the production of ammonia. In addition, failure of mechanisms to remove ammonia in the body would also contribute to increase in blood ammonia levels. As liver is the major site of ammonia removal in the body, ammonia levels in the liver were measured and more or less changes similar to those in serum were observed in liver (Fig. 2 c). This increase in liver ammonia level might be due to decreased detoxification of ammonia to urea in this tissue due to metabolic derangements leading to necrosis of liver cells. Hence, in thioacetamide induced liver failure, ammonia that enters the liver escapes the detoxification process and large amounts of ammonia enters the systemic circulation. The increase in liver ammonia level is known to be reflected in elevated serum ammonia levels in liver failure conditions (Bruck *et al.,* 1999; 2002). In the present study also the changes in liver ammonia levels are faithfully reflected in the serum. This is supported by the correlation coefficient of 0.84 between liver and serum ammonia levels (Fig. 3 b). Once ammonia enters systemic circulation, it will flood the tissue such as brain. Such an elevation in **the** brain ammonia levels is well known to be a hallmark of this condition (Albrecht *et al.,* 1990; Norenberg 1998).

Fig. 3: Correlation between (a) serum-brain (b) serum-liver ammonia levels in rats with TAA-induced FHF

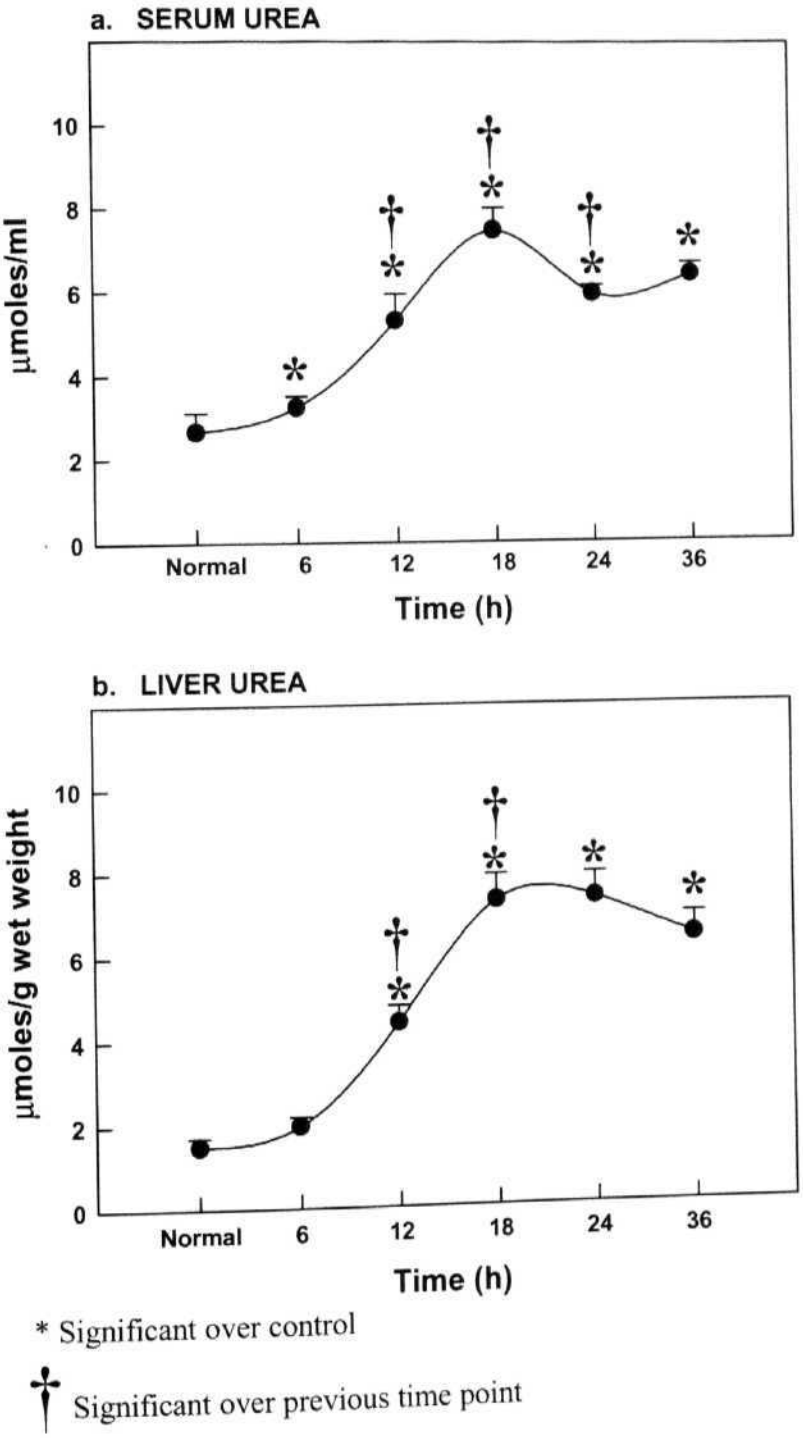


Results of the present study also indicate a progressive increase in brain ammonia levels in thioacetamide induced fulminant hepatic failure (Fig. 2b). Moreover, a close correlation (r^2 = 0.97) was seen between serum and brain ammonia levels (Fig. 3a). Increased levels of ammonia in brain has been considered as the chief culprit in the neurological dysfunction observed in fulminant hepatic failure (Albrecht 1998; Butterworth *et al.,* 1987; Norenberg 1996; 1998).

3.5.3 Urea

To verify the tenet that increased ammonia levels in the serum and in liver are due to deranged detoxification of ammonia to urea, levels of urea were measured both in serum and in liver. Serum (Fig. 4a) and liver (Fig. 4b) urea levels increased significantly in the rats administered with thioacetamide. In serum, there was a progressive elevation in urea levels till 18 h. By this time period there was a 2.8 fold increase over the controls. There was a 2 fold increase of urea level at 12 h time period after thioacetamide injection. In liver, the urea level was elevated by 3 folds at 12 h and 5 folds at 18 h after thioacetamide injection and remained same even after 24 h. In summary, urea levels increased both in serum and in liver progressively up to 18 h after the administration of thioacetamide with slight decrease thereafter. These results indicated that the hepatocytes that have survived the toxic effects of thioacetamide have increased the synthesis of urea, which reached the peak level by 18 h.

Fig. 4: Urea levels in the (a) serum and (b) liver of normal and thioacetamide treated rats at various time points



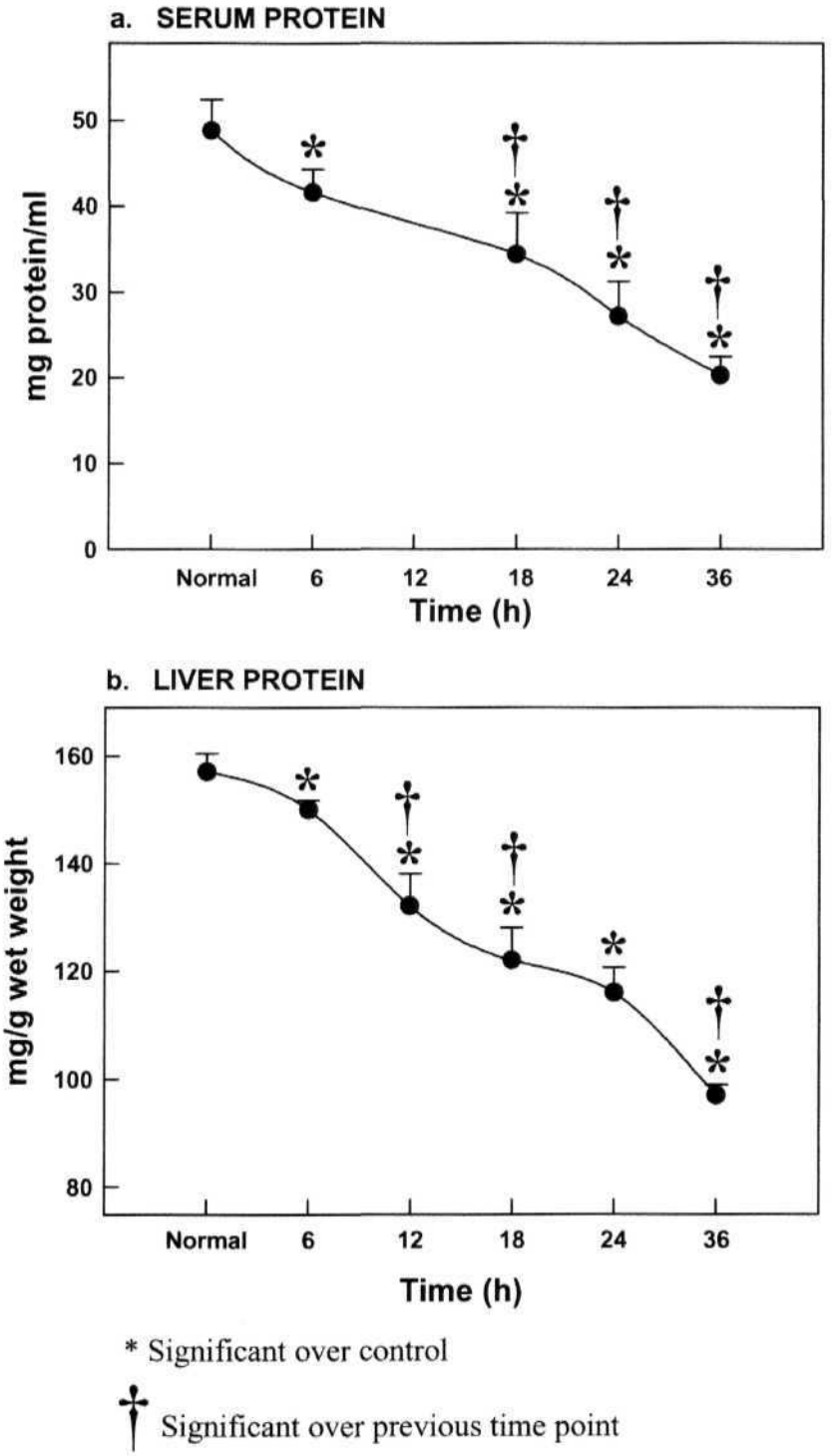
This might reflect the maximal capacity of the surviving hepatocytes (the number of which might be dwindling with time) to synthesize urea and explains lack of increase in the urea levels thereafter (Haussinger *et al.,* 1984).

3.5.4 Proteins

As liver is the major contributor to serum proteins, protein levels were also measured in the liver and in the serum of the animals treated with thioacetamide. The decrease in the liver protein (Fig. 5b) content by 6 h was marginal but statistically significant. At 18 h and 24 h the levels of protein were reduced by almost 25% of the control value. By 36 h a major change of about 40% reduction was observed.

The decrease in the serum protein content (Fig. 5a) was significant at all the time intervals studied. At 18 h there was 30% decrease, 44% at 24 h and by the end of 36 h the levels were decreased by 60%. The decrease in serum protein in thioacetamide induced rat was not only significant in comparison to control but also with respect to previous time periods. The decrease in protein content in liver may be due to the failure of liver to synthesize proteins due to the loss of hepatocytes by way of thioacetamide induced necrosis. Leakage of protein can also be a factor for the decreased protein content of the liver. The decrease of protein content in serum, however, does not support such a possibility.

Fig. 5: Levels of (a) serum and (b) liver protein in normal and thioacetamide treated rats at various time points



These results suggest the possible impaired protein synthesis in the liver of thioacetamide treated rats, leading to decreased serum protein levels. As the proteins contribute substantially to the osmotic pressure of serum, loss of proteins would decrease the osmolality of the serum. This might lead to increased load on the kidneys and also edema of other tissues (Reeba, 1995).

3.5.5 Aminotransferases

One of the standard methods to assess liver function in clinical conditions is to measure the activities of aminotransferases (transaminases) in the serum after the onset of liver failure. Such an effort was also made in the present study. An eight fold increase in serum alanine aminotransferase (ALT) was seen at 36h after the administration thioacetamide (Fig. 6a). A corresponding decrease was seen in the activity of this enzyme in the liver tissue under these conditions (Fig. 6b). Similarly, a three fold increase in the activity of serum aspartate aminotransferase (AST) was observed at 36 h after the administration of second dose of thioacetamide (Fig. 7a) while a 10 fold decrease was seen in the activity of this enzyme in the liver (Fig. 7b). Severe liver injury manifested by the elevation of serum AST and ALT were reported in thioacetamide insulted rats. (Norton *et al.,* 1997; Bruck et al., 1999; 2002). A close correlation with a r^2 value of 0.967 and 0.935 was observed between the changes in liver and serum activities of ALT and AST enzymes respectively (Fig. 8a, 8b).

Fig. 6: Activities of alanine amino transferase in (a) serum and (b) liver of normal and thioacetamide treated rats at different time points

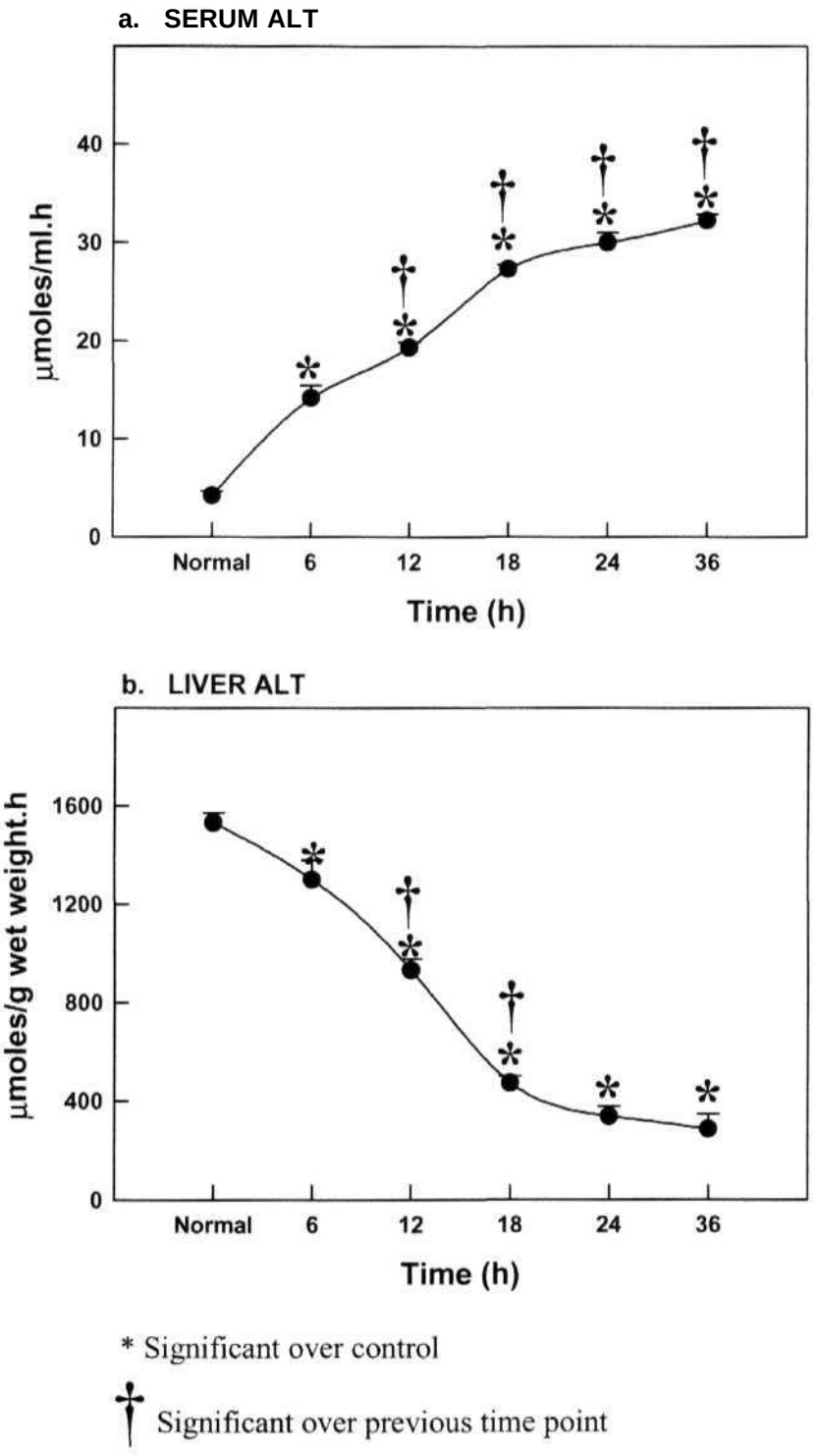


Fig. 7: Activities of aspartate amino transferase in (a) serum and (b) liver of normal and thioacetamide treated rats at different time points

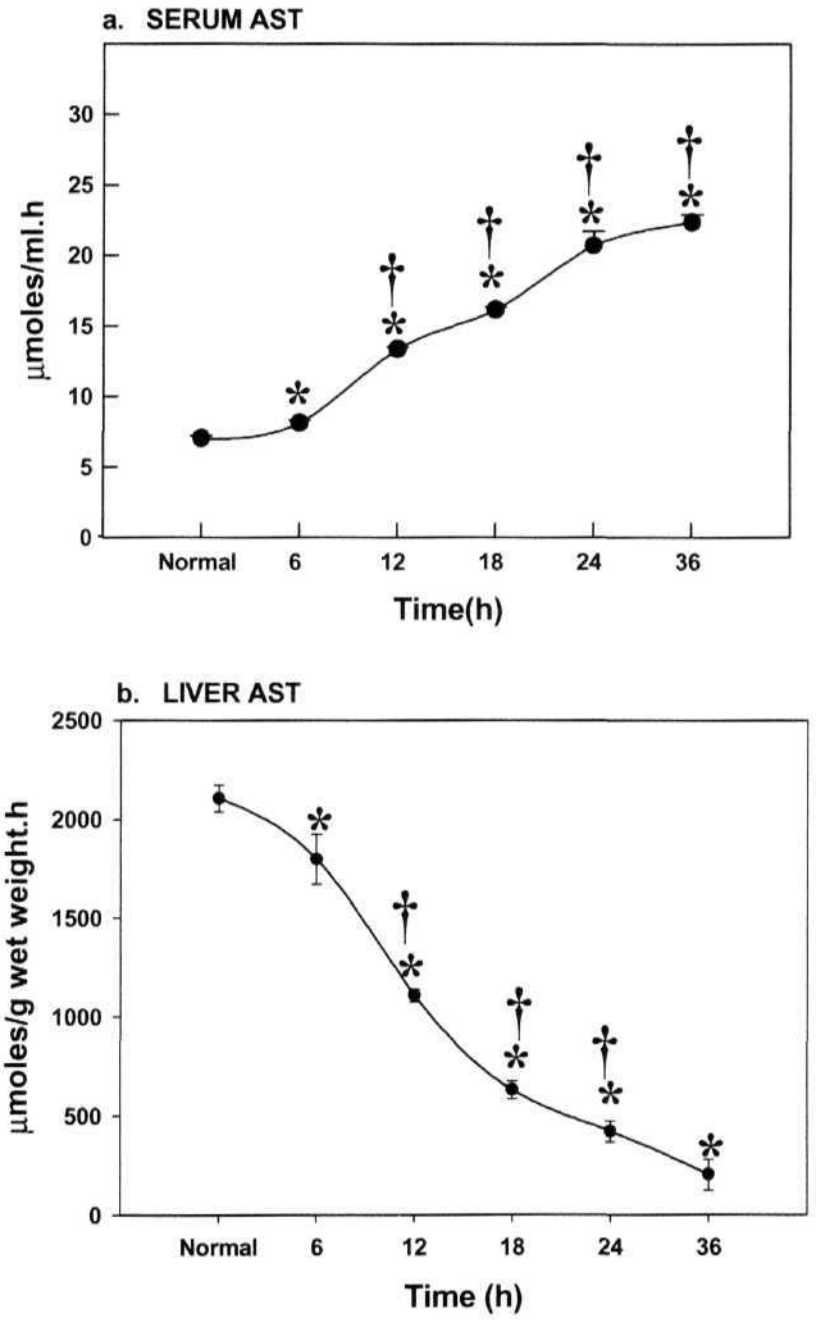
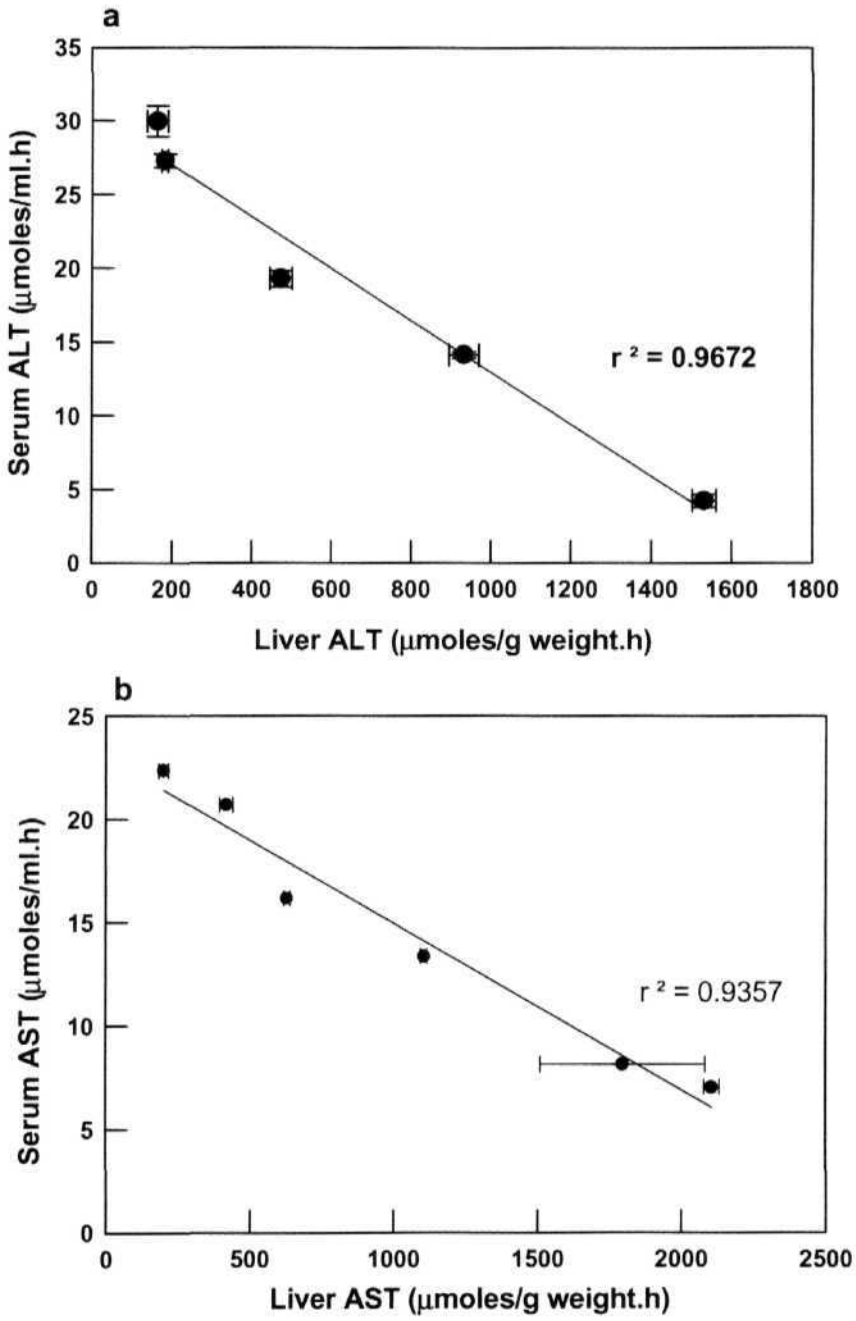


Fig. 8: Correlation between
(a) Serum and liver alanine aminotransferase and
(b) Serum and liver aspartate aminotransferase in thioacetamide treated rats



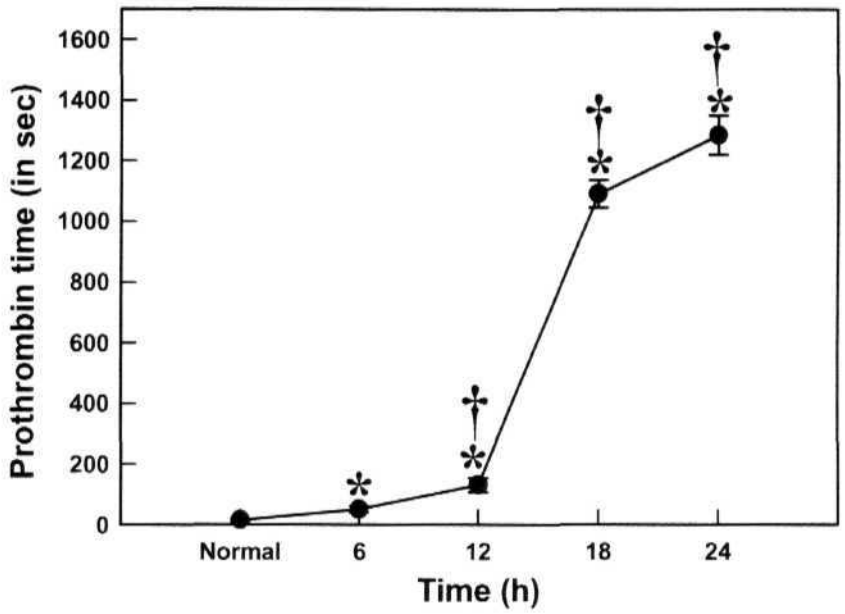
The increase in the activities of these enzymes in the serum might be due to the necrosis and subsequent release of these enzymes from the hepatocytes into the blood.

3.5.6 Prothrombin Time

During the standardization of the animal model it was observed that the time required for the clotting of the blood was increasing with increasing time after the administration of thioacetamide into the rats. In order to understand the possible factors, prothrombin time (PT) was measured in the rats treated with thioacetamide. Following the administration of thioacetamide, the prothrombin time increased progressively, reaching a maximum at 24 h (Fig. 9). A three fold increase in PT was observed as early as 6 h and 9 fold at the end of 12 h after the administration of thioacetamide. The PT was further increased to 17 min and 21 min at 18 and 24 h respectively. Significantly prolonged prothrombin time was also reported by Bruck and coworkers in similar conditions of thioacetamide treated rats (Bruck *et al.,* 1999).

The clotting of blood depends on the primary platelet plug formed along with the formation of a stable fibrin clot. Liver is the primary site of production of Fibrinogen and Prothrombin. The latter factor plays a crucial role in initiating the cascade of blood clotting reactions. Prothrombin is converted to thrombin in the presence of Ca^{++} and thrombin converts fibrinogen to fibrin.

Fig. 9: Changes in the prothrombin time in normal and thioacetamide treated rats



As prothrombin is synthesized and secreted into the blood by liver, any damage done to the liver will be reflected clearly on the levels of prothrombin in the blood and thus on the time required for clot formation by the blood.

The foregoing studies thus clearly indicate impaired function of liver in the thioacetamide-induced rats, suggesting possible structural alterations in the liver tissue. In order to test the effect of TAA administration on the liver tissue damage further histopathological studies were undertaken.

3.6 Histopathology

In order to study the histopathological studies of the TAA-induced liver, rats were sacrificed after the indicated time periods (0,6,12,18,24 h) after the second dose of administration of the drug. The liver specimen was fixed in Bouin's fluid, embedded in paraffin wax, sliced the sections, stained the sections with haematoxylin and eosin and observed under light microscopy. The photomicrographs of the liver tissue of normal rats show the presence of normal hepatocytes with distinct nuclei (Fig. 10). After 6 h of thioacetamide administration, the photomicrographs of the liver showed the presence of necrotic hepatocytes with darkly stained nuclei that are mainly concentrated around the capillary. The cells away from the capillary still show distinct, spherical and normal nuclei (Fig. 11). At 12 h after the administration of the drug, extensive patches of necrotic cells with highly condensed nuclei are noticed. The cells away from the capillary also show necrosis, indicating the increased effect of the injected thioacetamide. Changes in the shape of the nucleus is also noticed (Fig. 12). Fig. 13, 14 show the photomicrographs of the tissue at 18 and 24 h after the administration of thioacetamide respectively. All most all the cells are degenerated and maximal condensation of the cellular mass and nucleus condensation is observed here.

Results of the present study clearly established the potency of thioacetamide in inducing fulminant hepatic failure conditions in rats.

Having established the animal model for FHF, attention is now focused on the involvement of the nonsynaptic mitochondria in the pathophysiology of the disease. For this purpose, contamination-free nonsynaptic mitochondria were isolated by density gradient centrifugation and used for studying the role of cerebral mitochondrial dysfunctions in the etiology of FHF.

Fig. 10: Photomicrographs showing the liver cells from the control rats. These cells show the presence of normal hepatocytes with distinct nucleus. a : Lower magnification (30 μm = 1.025 cm); b : Higher magnification (10 μm = 1.5 cm)

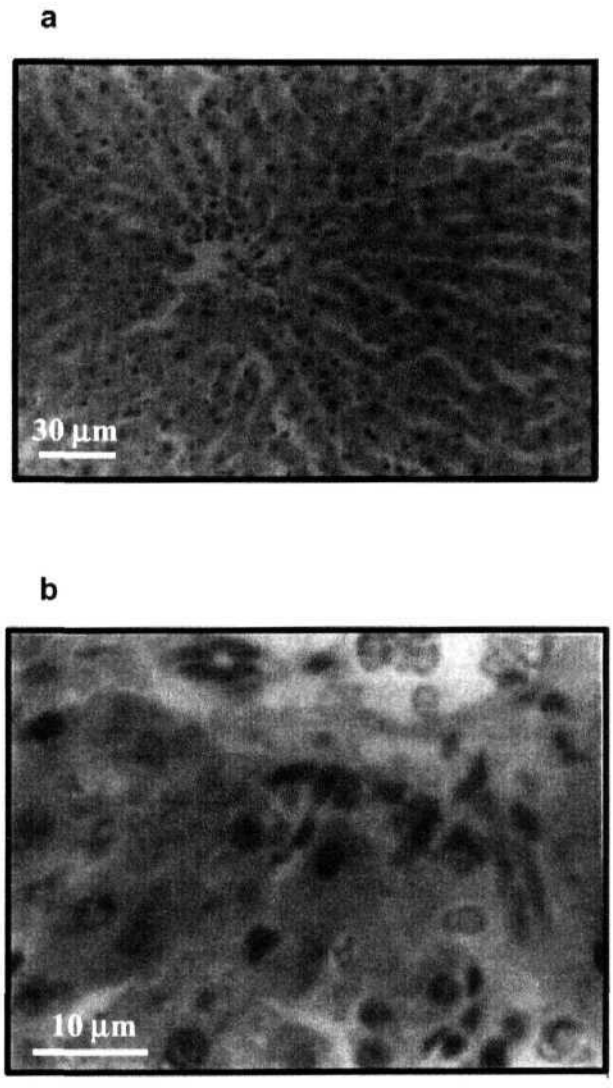


Fig. 11: Photomicrographs of liver taken from the rat after 6 h thioacetamide treatment. The micrograph shows the presence of necrotic (degenerating) hepatocytes, which are mainly concentrated around the capillaries (blood vessels). (→) shows the presence of a capillary. Fairly condensed nuclei that are darkly stained in the necrotic hepatocytes while the surrounding cells, which are away from the damaged region, still show large spherical nucleus. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)

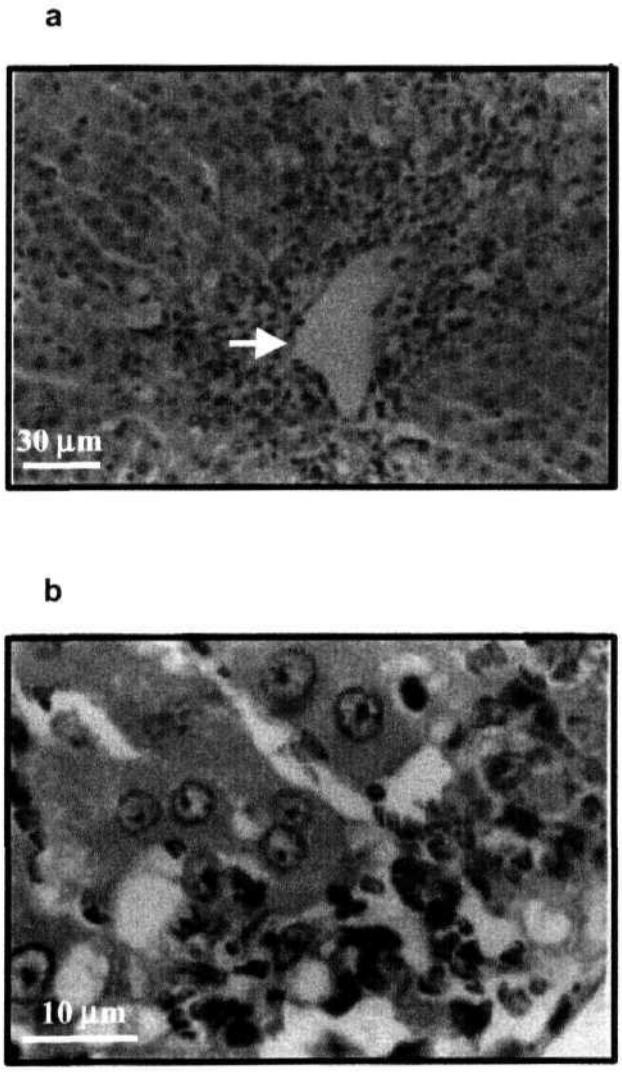


Fig. 12: These micrographs show the prolonged effect of thioacetamide on liver cells after 12 h after the treatment with the drug. The presence of big patches of degenerating cells with highly condensed nucleus in the hepatocytes are noticed here. These patches are present near the capillary (→) and also away from the capillary indicating the increased area of liver degeneration. The changes in the nucleus shape are clearly noticed. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)

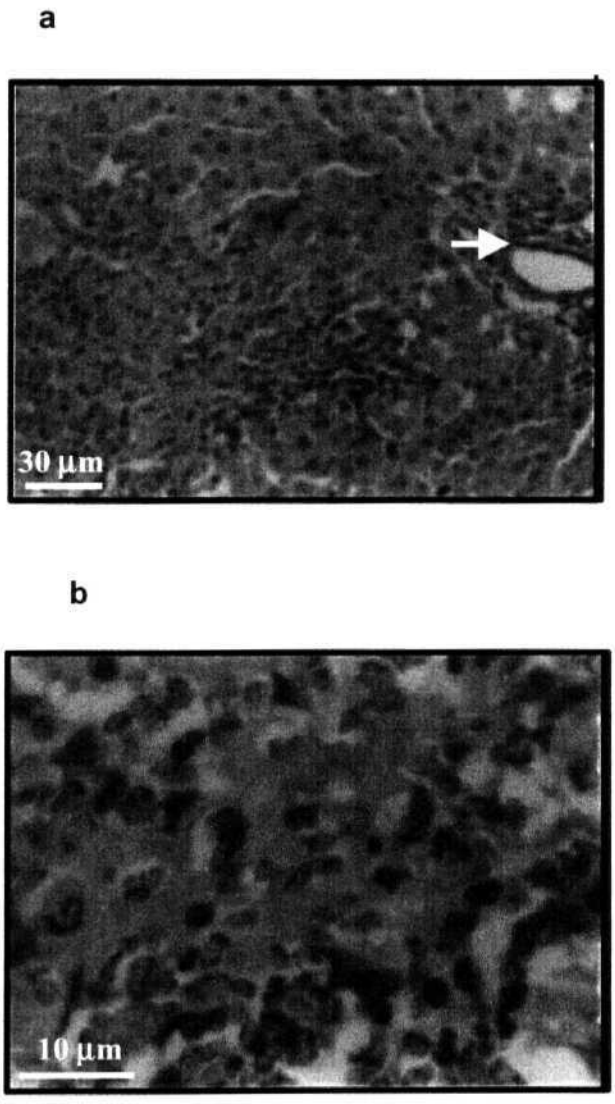
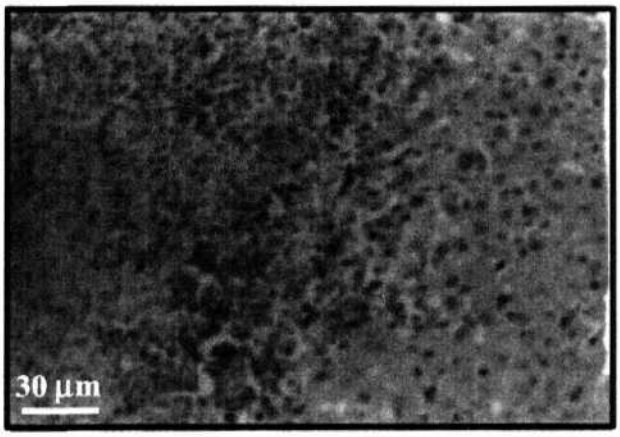


Fig. 13: Micrographs showing the effect of thioacetamide at the end of 18 n of treatment after the second dose. With the longer treatment of thioacetamide there was a significant increase in the degeneration of the liver. Due to the higher degree of necrosis, well-condensed nuclei are observed in this preparation. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)

a



b

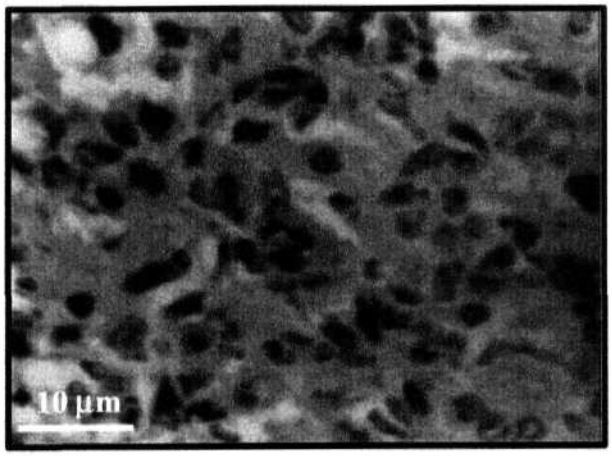
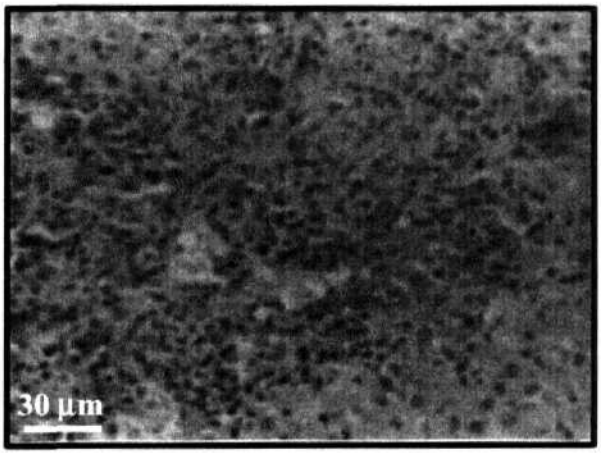
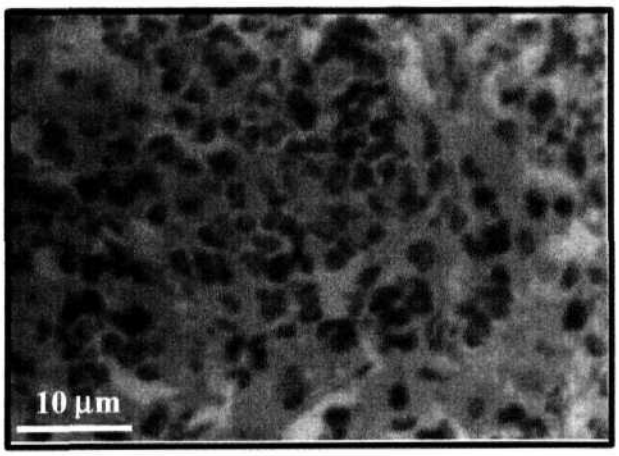


Fig. 14:The micrographs here shows the highest degree of damage done to the liver at the end of 24 h after the treatment with the drug. Almost all the liver cells are degenerated. Maximal condensation of the cellular mass apart from the condensation in the nucleus can be seen. It is difficult to find normal hepatocytes at this time point after the drug treatment. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)

a



b



TAA-induced FHF: Effects on cerebral nonsynaptic mitochondria

4.1 Standardization of the Enzyme Assays

Before performing the enzyme assays, basic protocol experiments were carried to determine optimal concentrations of the enzyme protein, cofactors, substrates to be used, optimal incubation period (wherever necessary) for each enzyme in the mitochondria enriched preparations. From these studies, an appropriate quantity of protein, cofactors and substrates were selected and used for the routine assays of the enzymes.

4.2 Preparation of Nonsynaptic Mitochondria

Preparation of mitochondria from brain tissue is slightly complicated due to the presence of myelin sheath and nerve terminals in the brain. During homogenization nerve terminals are sheared from axons and form vesicles which have density closer to that of mitochondria. As per the convention these pinched off vesicles were designated as the synaptosomes. Similarly, the shearing forces generated in the process of homogenization also tear off myelin sheath from the axon and form vesicles which have a density closure to that of mitochondria. In differential centrifugation these three subcellular fractions i.e., mitochondria, synaptosomes and myelin sediment at the same centrifugal force (12000g). They cannot be separated from each other by this centrifugal technique.

Density gradient centrifugation is usually adopted to separate mitochondria from nerve terminals and myelin vesicles. The most commonly used density gradient media is sucrose. In this process,

mitochondria sediment as a pellet in 1M sucrose solution, synaptosomcs forming an interface between 0.8 and 1M sucrose. Myelin floats on the surface of the top layer of 0.32M sucrose. This method yields pure mitochondria free from the other two contaminants. However, several studies have shown that mitochondria exposed to 1 M sucrose which is hyper osmotic, and subsequently to isosmotic sucrose, solution alters several of the membrane permeability properties of the mitochondria. This sucrose density gradient was used to asses the activities of the citric acid cycle enzymes since the determination of these enzyme activities do not require well coupled intact mitochondria. Further, we use Triton X -100, a detergent that ruptures the mitochondrial membranes, for making the substrates available for the enzymes. In the later part of the study which required intact and well coupled mitochondria Ficoll-400 density gradient was used.

4.3 Assessment of the Purity of the Isolated Subcellular fractions

In order to carry out the studies involving mitochondria, a subcellular fraction, it is very much necessary to determine the purity of the isolated fraction and the extent of cross contamination with other subcellular fractions. Usually microscopic and biochemical methods are used for the assessment of the purity of the fractions obtained. Electron microscopy is generally used since the resolution of the light microscope is not adequate to study the subcellular fractions. This procedure involves large amount of time in processing of the sample

and thus it restricts the regular usage of electron microscope for the determination of the purity. Hence biochemical methods which have been well developed have been used in the present study. For this purpose, activities of the marker enzymes, whose subcellular localization have already been established were assessed. The activity of Succinate dehydrogenase (SDH), an enzyme located in the inner mitochondrial membrane was used as a marker for the assessment of the mitochondrial purity (Singer *et al.,* 1972). Glutamic acid decarboxylase (GAD) was used as a marker for the synaptosomcs (Salganicoff and De Robertis, 1965). The mitochondria present in these synaptosomes are called synaptic mitochondria. In the present study the term mitochondria refers to the one that sediments independent of the synaptosomes, which are referred to as non-synaptic mitochondria.

4.3.1 Assay of Succinate Dehydrogenase (SDH): Marker for mitochondria

In the mitochondrial fraction isolated by the sucrose density gradient, the activity of the mitochondrial marker, SDH, was found to be the highest in the nonsynaptic mitochondria (Fig. 15a). The activity of the marker SDH in the synaptosomal fraction was found to be 21% of the activity found in the mitochondrial fraction (Fig. 15a). This activity may be due to the presence of synaptic mitochondria. Myelin fraction had a negligible amount of activity when compared to the other two fractions (Fig. 15a). This indicates that the mitochondrial fraction is

relatively pure from the contamination with other two subcellular fractions.

The studies on mitochondria prepared by the Ficoll density gradient also revealed that the mitochondrial fraction isolated is pure and free from contamination. The mitochondria isolated by ficoll-400 gradient also recorded the highest activity of SDH while synaptosomes had 11% and myelin had just 0.7% of the activity of the mitochondrial fraction (Fig. 16a).

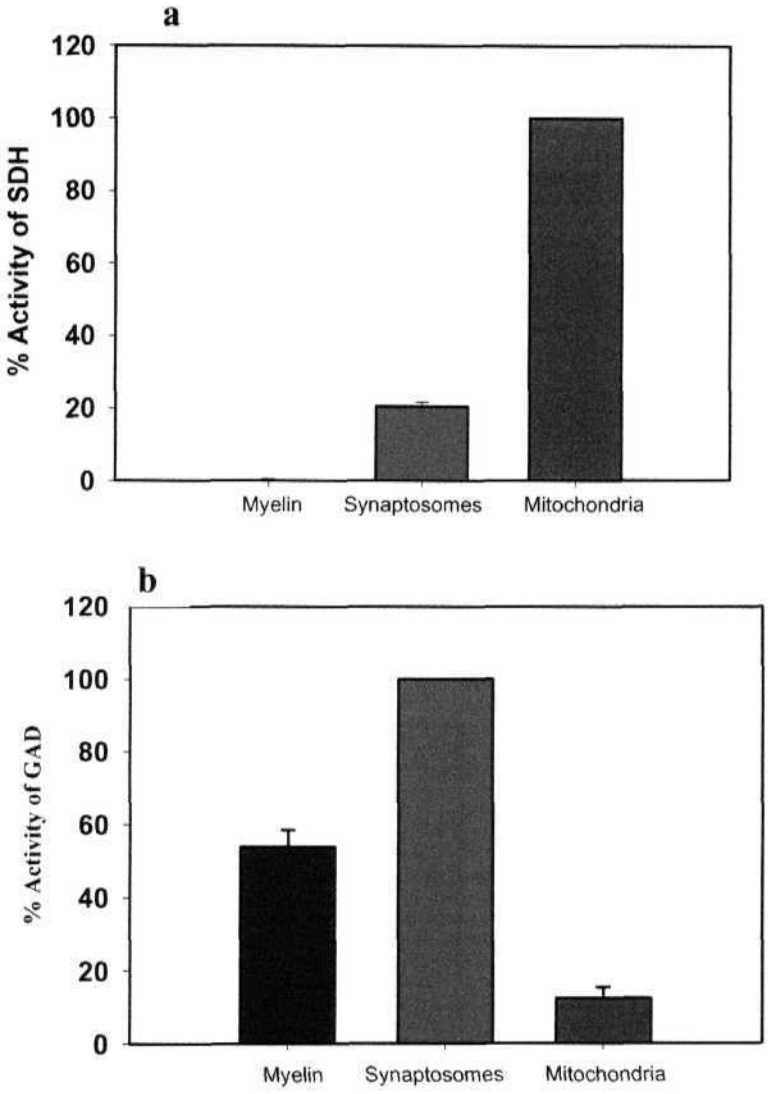
4.3.2 Assay of Glutamic Acid Decarboxylase (GAD): Marker for synaptosomes

Synaptosomal marker, GAD activity was found to be the highest in the synaptosomal fractions isolated by both sucrose and ficoll- 400 density gradient separations.

In various fractions isolated by the sucrose density gradient, the activity of the GAD was found to be the highest in the synaptosomal fraction. The activity of this marker in the mitochondria was found to be 12.5% of the activity found in the synaptosomal fraction (Fig. 15b). The GAD activity in myelin fraction was observed to be 54% of the synaptosomes (Fig. 15b). This activity could be due to the presence of soluble form of GAD which could have got separated in the myelin fraction. This indicates that the mitochondria isolated are relatively free from contamination with the other fractions.

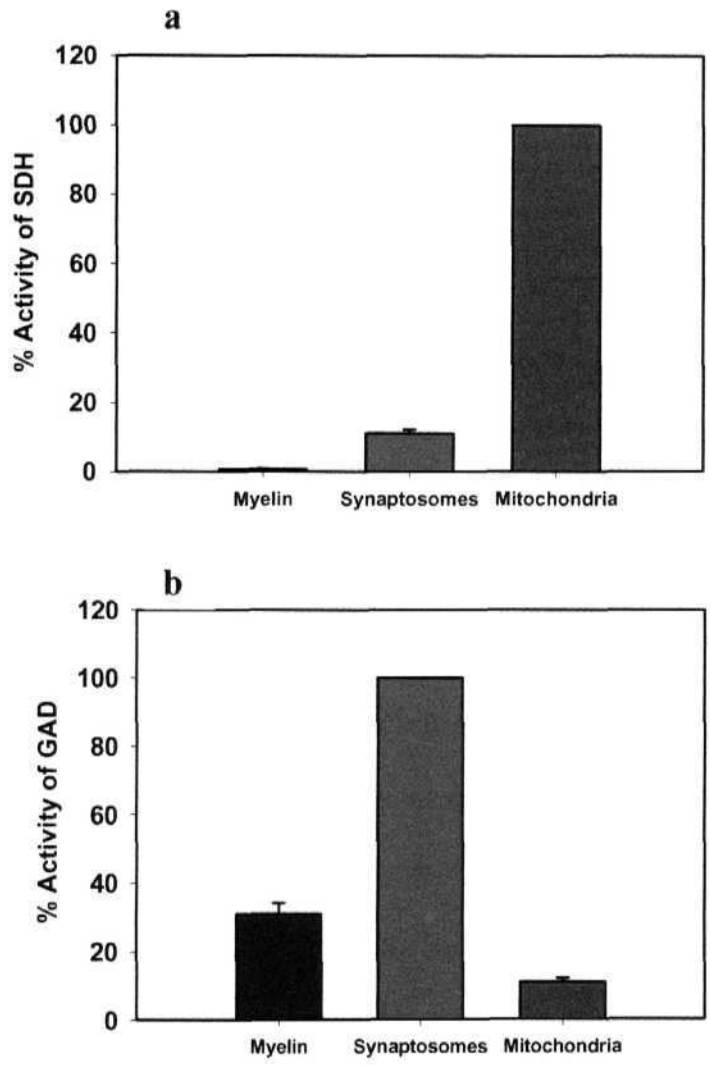
Similar results were obtained with the ficoll- 400 based isolation of subcellular fractions. When this method was adopted synaptosomes

Fig. 15: Assessment of purity of the subcellular fractions isolated by sucrose density gradient



Activity of (a) succinate dehydrogenase (b) glutamic acid decarboxylase in different subcellular fractions (myelin, synaptosomes, and mitochondria) isolated by sucrose density gradient. For SDH marker, its activity was taken as 100% in mitochondria and the activity of other fractions was expressed as relative % activity of mitochondria. For GAD marker, activity of GAD in synaptosomes was considered as 100% and the activity of other fractions was expressed as relative % activity of synaptosomes.

Fig. 16: Assessment of purity of the subcellular fractions isolated by Ficoll-400 density gradient



Activity of (a) succinate dehydrogenase (b) glutamic acid decarboxylase in different subcellular fractions (myelin, synaptosomes, and mitochondria) isolated by Ficoll-400 density gradient. For SDH marker, its activity was taken as 100% in mitochondria and the activity of other fractions was expressed as relative % activity of mitochondria. For GAD marker, activity of GAD in synaptosomes was considered as 100% and the activity of other fractions was expressed as relative % activity of synaptosomes.

had the highest activity while mitochondria had 11% and myelin had 31% of the activity of synaptosomes (Fig. 16b) indicating that all the three fractions are being isolated separately without getting co-separated with the other fractions.

4.4 Impact of Thioacetamide-Induced FHF on the Functional Activity of Mitochondria

Elevation of brain ammonia levels is known to be neurotoxic. The mechanism of ammonia toxicity, by itself, is a topic of much controversy and debate. Since the literature survey discussed in the introduction suggests a possible role of mitochondria involvement in the FHF conditions, experiments were designed to study the alterations in the functions of cerebral mitochondria.

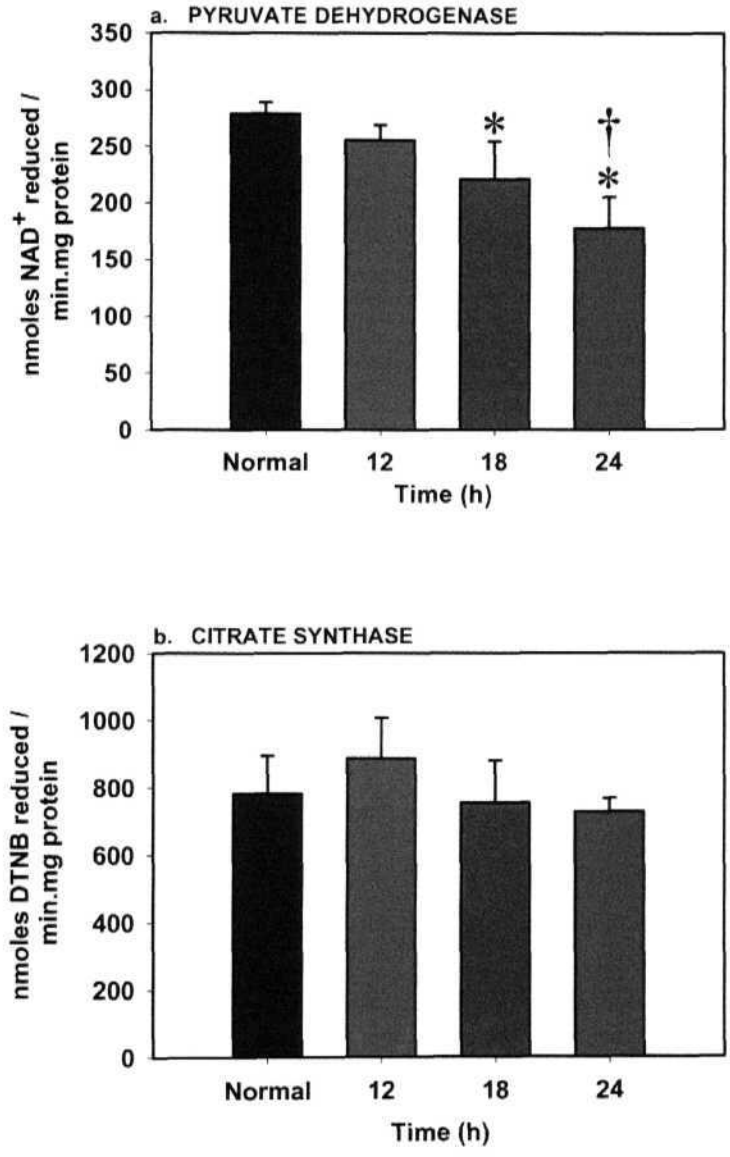
4.4.1 Citric Acid Cycle Enzyme Activities

For the assay of TCA cycle enzymes the mitochondria were isolated in sucrose density gradient, washed thoroughly and suspended in isotonic sucrose media. They were treated with Triton X - 100 to lyse the membranes in order to make the enzymes to have sufficient accessibility for the substrates.

The activity of pyruvate dehydrogenase (PDH) was determined by following the decrease in absorbance at 340nm. The activity of Pyruvate dehydrogenase showed a decrease of 8.2% by the end of 12 h and then by 20.7% by 18 h. At the end of 24 h the PDH recorded its lowest activity with a decrease of 36% over the control (Fig. 17a). The activity of Citrate synthase did not show any significant alterations in its

activity at any of the time periods after the administration of the drug. Though there was an initial increase followed by a decrease, the change was not statistically significant (Fig. 17b). The activity of Isocitrate dehydrogenase did not show any statistically significant change in its activity, though there was a slight increase at 18h interval (Fig. 18a). At 18 h the activity of a-ketoglutarate dehydrogenase was elevated by 34.7% and at 24h the increase in its activity was 27% (Fig. 18b). The change in the activity at 18 h was significant over both control as well as over the previous time point. Succinate dehydrogenase recorded a significant decrease in the activity at all the time points after the administration of the drug. This enzyme showed a reduction of 15% and 31% over the control at 12 and 18 h respectively (Fig. 19a). At 24 h the decrease was 23% in the activity of SDH. Malate dehydrogenase, in the direction of oxaloacetate formation, did not show significant change in the activity at all the time periods studied after the thioacetamide administration (Fig. 19b). The activity was more or less the same at all the time periods of treatment.

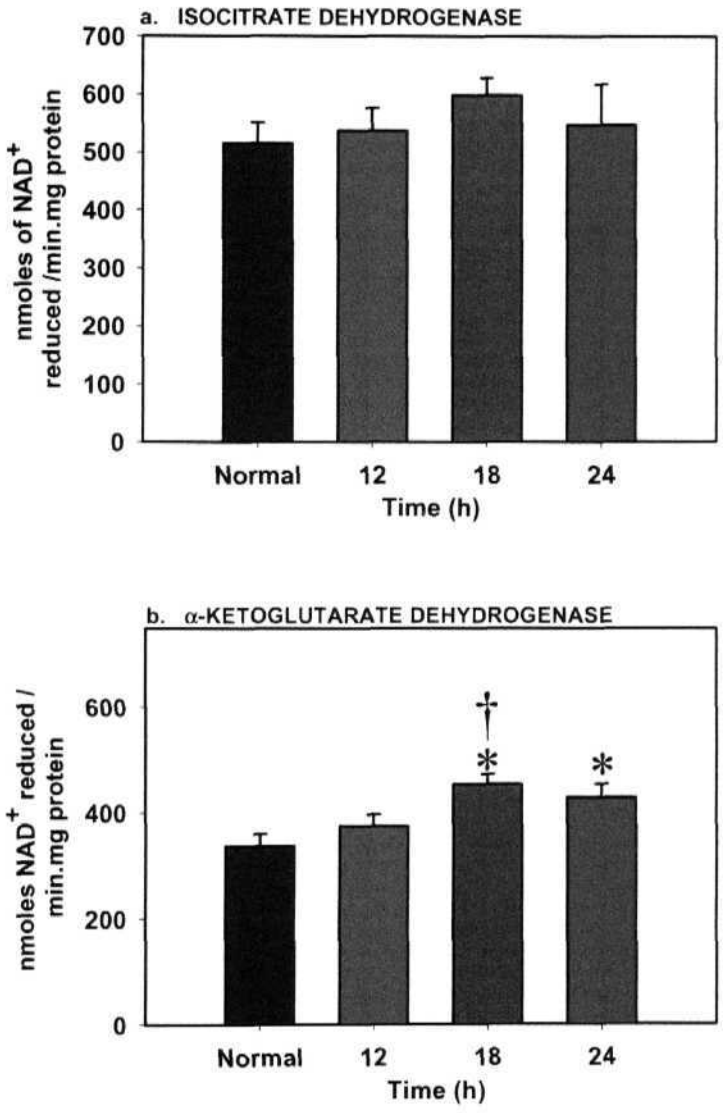
Fig. 17: Activities of pyruvate dehydrogenase and citrate synthase



Changes in the activities of (a) pyruvate dehydrogenase and (b) citrate synthase in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.

* Significant over control. † Significant over previous time point

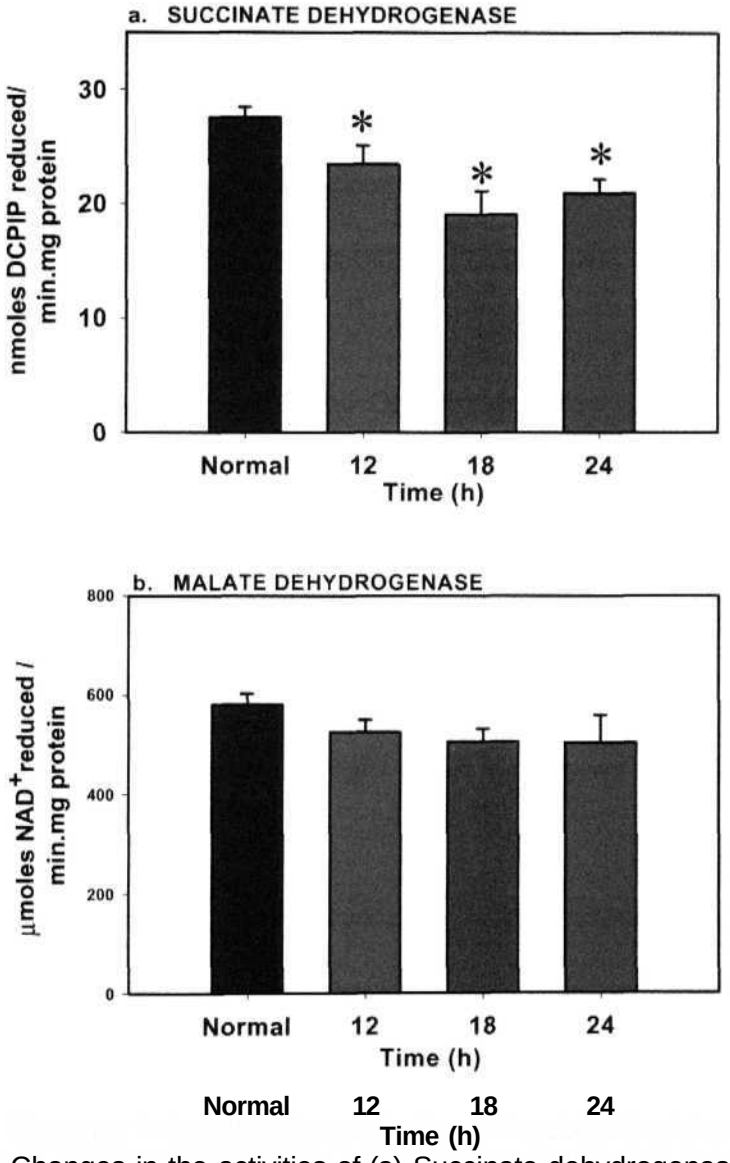
Fig. 18: Activities of isocitrate dehydrogenase and a- ketoglutarate dehydrogenase



Changes in the activities of (a) isocitrate dehydrogenase and (b) a- ketoglutarate dehydrogenase in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.

* Significant over control. † Significant over previous time point

Fig. 19: Activities of Succinate dehydrogenase and Malate dehydrogenase in brain mitochondria



Changes in the activities of (a) Succinate dehydrogenase and (b) Malate dehydrogenase in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.
* Significant over control.

4.4.2 Effect of Thioacetamide-Induced FHF on the Activities of Electron Transport Chain Enzymes

4.4.2.1 NADH - Ubiquinone Oxidoreductase (Complex I)

The decrease in the absorbance due the oxidation of NADH at 340nm leading to the reduction of ubiquinone (CoQ_1) to ubiquinol was measured. The activity of Complex I showed a decrease of 31% at 18h and 21% at 24 h in the thioacetamide treated rats in comparison with normal rats (Fig. 20a).

4.4.2.2 Succinate - Ubiquinone Oxidoreductase (Complex II)

The disappearance of the colour of the DCPIP dye due to the secondary reduction of the dye by the ubiquinol that is formed as a result of the reduction of the ubiquinone (C0Q2) compound was measured at 600nm.The activity of complex II showed no significant alteration in the experimental rats when compared to those in control rats (Fig. 20b).

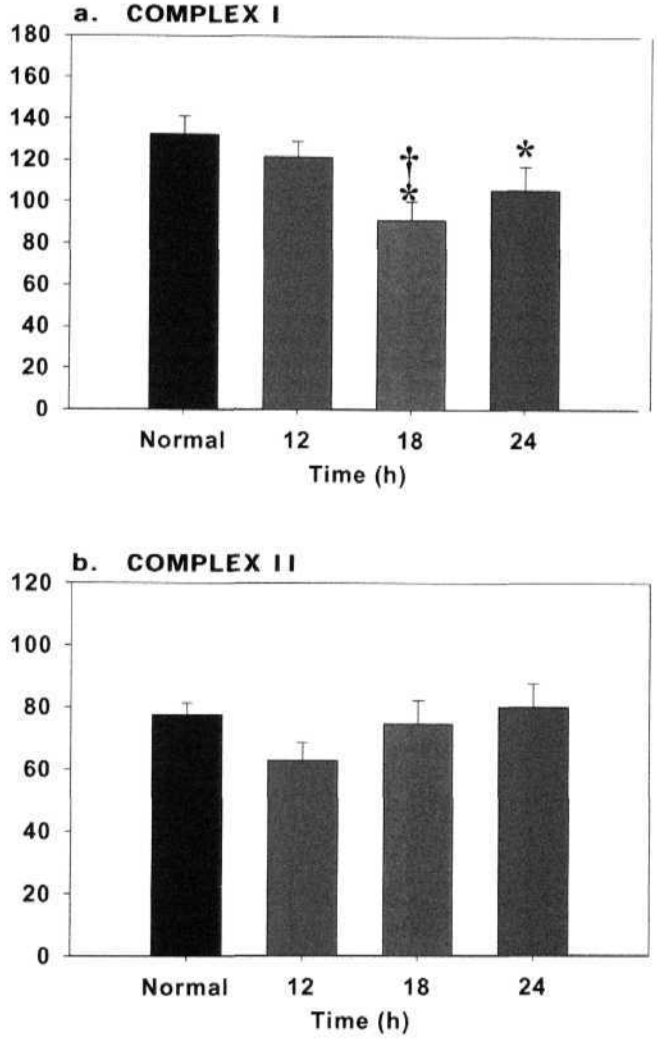
4.4.2.3 Ubiquinone-Cytochrome-c Oxidoreductase (Complex III)

Complex III donates the electrons from ubiquinol to cytochrome *c,* thus resulting in the reduction of cytochrome *c.* This reduction of the cytochrome c was measured at 550nm with a reference wave length of 580nm.The activity of complex III exhibited no statistically significant change in the activity at 12 h. But a very significant reduction in the enzyme activity was observed at later periods of time. Its activity was lowered by 22% at 18 h by 29% by 24 h in the TAA-induced FHF rats when compared to the control rats (Fig. 21a).

4.4.2.4 Cytochrome-c Oxidase (Complex IV)

The activity of Cytochrome-c oxidase was determined by monitoring the decrease in the absorbance at 550nm due to the oxidation of cytochrome c. Before the reaction was done, cytochrome c was reduced by the addition of a pinch of ascorbate. No significant differences were observed in the activity of respiratory complex IV at any of the time periods after the induction of FHF (Fig. 21b).

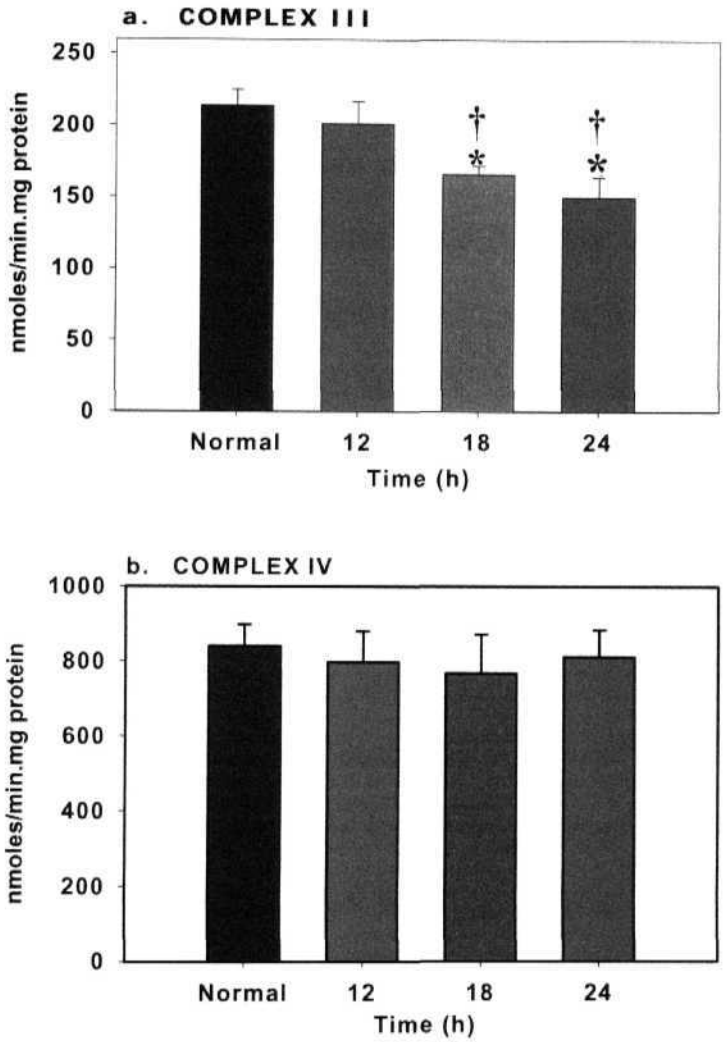
Fig. 20: Effect of thioacetamide-induced FHF on NADH-ubiquinone oxidoreductase and Succinate-ubiquinone oxidoreductase



Changes in the activities of (a) NADH-ubiquinone oxidoreductase (Complex I) (b) Succinate- ubiquinone oxidoreductase (Complex II) in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.

* Significant over control. † Significant over previous time point

Fig. 21: Effect of thioacetamide-induced FHF on Ubiquinone-cytochrome c oxidoreductase and Cytochrome c oxidase



Changes in the activities of (a) Ubiquinone- Cytochrome c oxidoreductase (Complex III) (b) Cytochrome c oxidase (Complex IV) in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.

* Significant over control. † Significant over previous time point

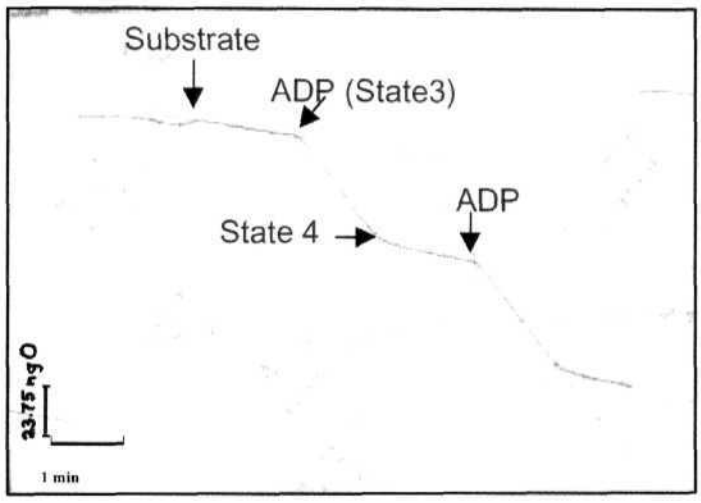
4.4.3 Measurement of Oxygen Consumption Studies in the Non-synaptic mitochondria.

Polarographic Method

In order to study the changes in the functioning of the mitochondria, mitochondrial respiration was measured polarographically with temperature controlled Clark oxygen electrode. For this purpose the intact coupled mitochondria were isolated by adapting ficoll-400 density gradient method. In this experiment state 3 (in the presence of ADP) and state 4 (in the absence of ADP) respirations were measured. For this purpose two different substrates for the respiratory complexes, pyruvate-malate and succinate were used.

The results of this study indicated that the changes in the mitochondrial respiration, when succinate was used as a substrate, were not statistically significant. An ideal curve indicating various states of the respiration has been shown in the figure (Fig. 22). In a typical assay, the reaction mixture contained sucrose, mannitol, KH_2PO_4, KCl, EDTA, and Tris-HCl, and the mitochondrial sample. The reaction was initiated by the addition of the substrate (succinate or pyruvate and malate). ADP was added to reaction mixture to measure the state 3 respiration and P/O ratio. The ratio of state 3 to state 4 was taken as the respiratory control ratio. The P/O ratio was calculated as the amount of ADP to that of the oxygen consumed during the state 3 respiration.

Fig. 22: A typical polarographic curve showing different stages of mitochondrial respiration



The typical assay mixture contained 25 mM sucrose, 75 mM mannitol, 5 mM KH_2PO_4 100 mM KCl, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4, in the presence of the respiratory substrate containing 0.5M succinate or 5 mM pyruvate and 2.5 mM malate. The actively respiring state in the presence of ADP is referred to as State 3 and slower respiration rate after all the ADP is exhausted is referred to as State 4.

The results of this experiment indicated that there was no change in the state 3 and state 4 respiration when succinate was used as the respiratory substrate (Fig. 23a, 23b). Hence no change was observed in the respiratory control ratio and in the P/O ratio (Fig. 23c, 23d). On the other hand, the state 3 respiration was lowered by 27% when Pyruvate–malate were used as the substrates (Fig. 23a). The respiratory control ratio was also found to be reduced by 22% in the mitochondria of the drug administered rats when pyruvate/malate were

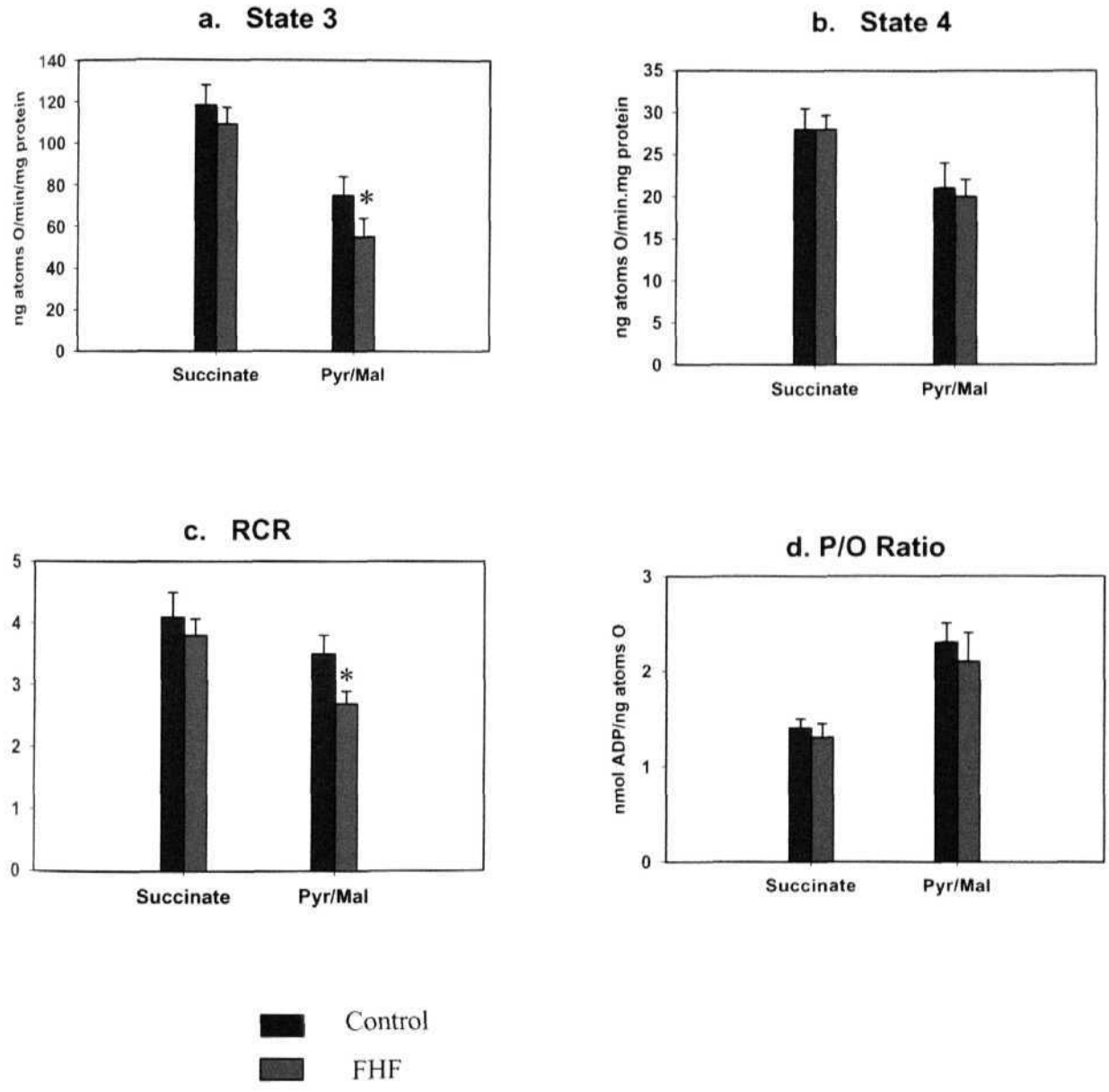
used as the substrates (Fig. 23c). Though the P/O ratio showed a decrease in pyruvate/malate induced respiration, it was not statistically significant (Fig. 23d).

4.5 Effect of Thioacetamide-Induced FHF on the Structural Integrity of the Nonsynaptic Mitochondria

Mitochondrial Swelling

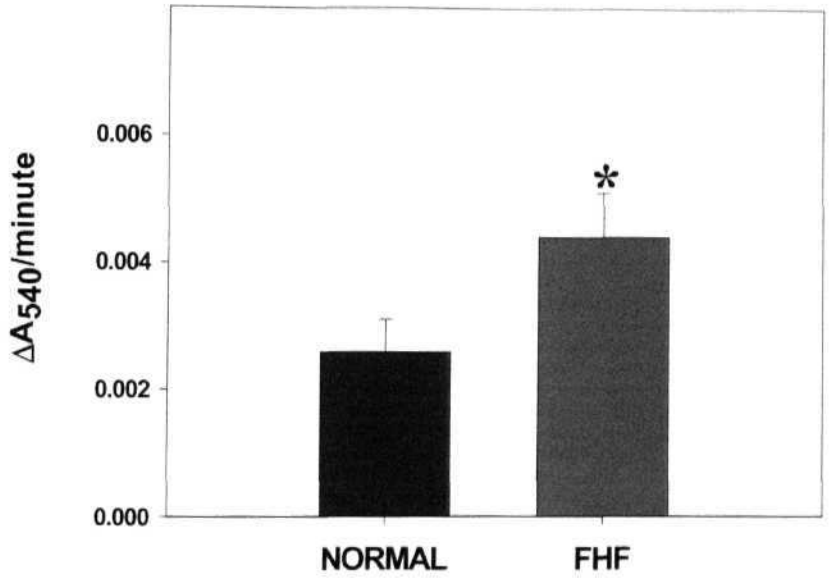
In order to see if there is any change in the structure of the mitochondria, changes in the volume of the mitochondria were determined by measuring the swelling of the mitochondria. The results of this study indicate that the volume of the mitochondria was significantly increased in the thioacetamide treated rats. The swelling studies performed by following the decrease in absorbance revealed a two fold increase in the volume in mitochondria (Fig. 24). This increase in the volume was further confirmed by the transmission electron microscopy. The photomicrographs of the TEM quite evidently reveal the changes in the mitochondria of the FHF induced rats (Fig. 25). The increase in the volume of mitochondria in the TAA-induced FHF rats (Fig. 25b) over the control (Fig. 25a) is indicated by the arrow mark. The magnified image of the mitochondria of Control (Fig. 25c) and drug administered rats (Fig. 25d) is also shown.

Fig. 23: Effect of thioacetamide induced FHF on mitochondrial respiration



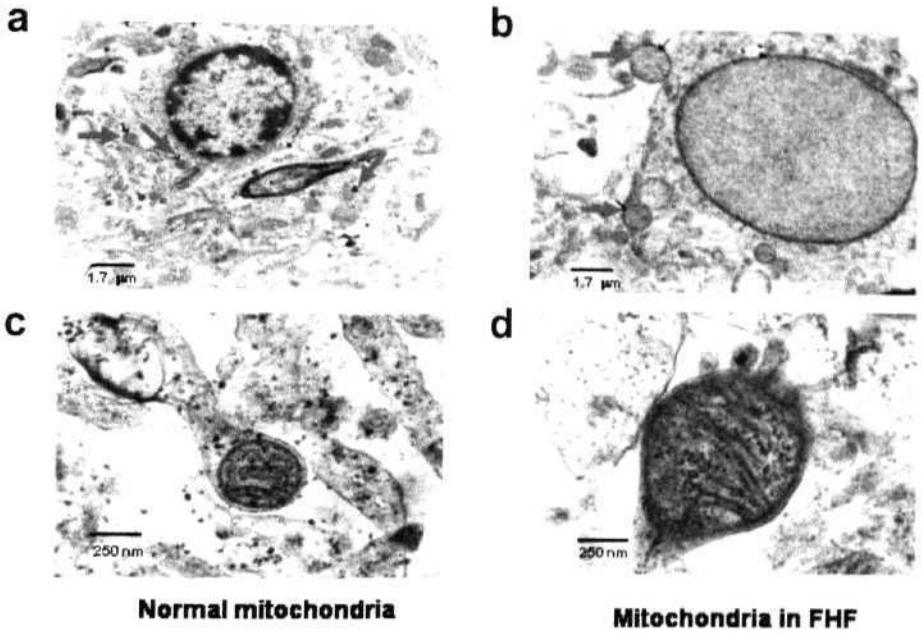
Changes in different parameters of the nonsynaptic mitochondrial respiration, (a) State 3 (b) State 4 (c) Respiratory control ratio (d) P/O ratio in control and thioacetamide induced FHF rats, 18h after the administration of the drug. Values are Mean ± Standard deviation of six sets of experiments.
* Significant over control.

Fig. 24: Mitochondrial swelling in thioacetamide induced FHF rats



Mitochondrial swelling was assessed by measuring the change in absorbance at 540nm in the nonsynaptic mitochondria of normal and FHF rats, 18 h after the administration of thioacetamide. * Significant over control.

Fig. 25: Transmission electron micrographs showing the swelling of mitochondria



Transmission electron micrographs of ultra thin sections of cerebral cortex of thioacetamide induced FHF rats at 18 h after the administration of the drug showing the volume changes in the mitochondria of normal (a & c), TAA induced FHF (b & d)

4.6 FHF Induced Oxidative Stress in the Mitochondria From Cerebral Cortex of Rats

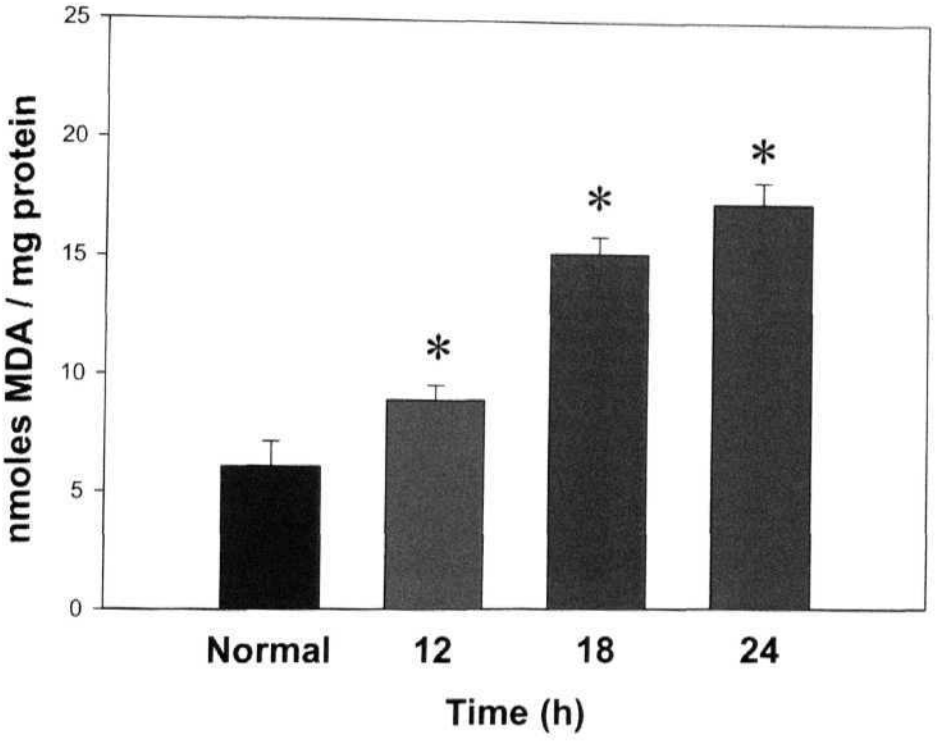
The results obtained during the assessment of the mitochondrial functions revealed that there are structural and functional dysfunctions of mitochondria in the FHF conditions. In search of reasons for these changes in the mitochondria, we have evaluated the involvement of oxidative stress on non synaptic mitochondria isolated from cerebral cortex in conditions of hepatic encephalopathy. This was done by studying changes in lipid peroxidation, total thiols and various antioxidant enzymes and other nonenzymatic parameters after inducing FHF by administering thioacetamide.

In the experimental animals with induced FHF, there was an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the nonsynaptic mitochondrial lipid peroxidation (Fig. 26). There was an increase in lipid peroxidation by 46% as early as 12 h after thioacetamide administration. At 18 h, it further increased by 140%, and then by 180% at 24 h after the administration of thioacetamide. The levels of total thiols, on the other hand, decreased by 14% at 12 h interval and 30% by 18 h when compared with the controls (Fig. 27). At 24 h, no further change was observed. There was no significant change in the activity of glutathione peroxidase at 12 h interval but there was a statistically significant decrease of 14% and 24% at 18 and 24 h, respectively (Fig. 28). The activity of glutathione reductase also decreased by 17% over the

controls, but only at 24 h (Fig. 29). There was an increase of 39% and 46% in the activity of Mn-SOD at 18 and 24 h after the administration of the thioacetamide, respectively (Fig. 30).

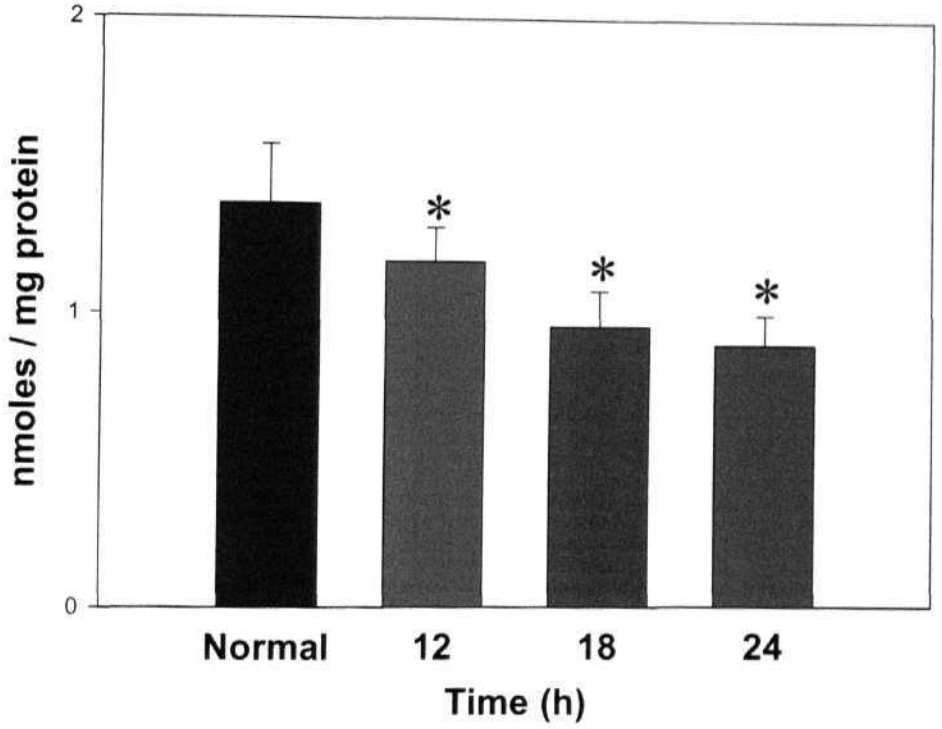
Further there was an increase of 44% in the levels of the reduced glutathione (GSH) content in the thioacetamide injected rats (Fig. 31). The oxidized form of glutathione (GSSG) was elevated by 2.7 folds in the TAA-induced FHF rats (Fig. 32). As a result of the changes in the GSH and GSSG levels the ratio of the GSH/GSSG was decreased by 52% in the rats affected with FHF (Fig. 33).

Fig. 26: Malondialdehyde levels in the brain mitochondria of FHF rats



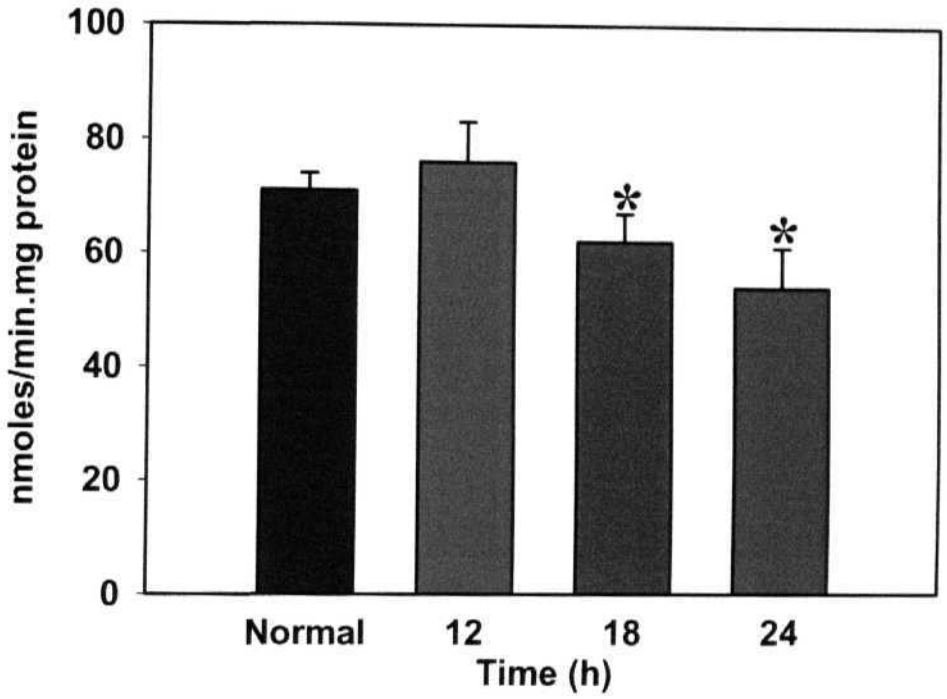
Changes in the malondialdehyde levels in the nonsynaptic mitochondria isolated from cerebral cortex of normal and thioacetamide-induced FHF rats at 12, 18 and 24h after the administration of the drug. All values are mean ± Standard deviation of six individual experiments done in duplicates.
* Statistically significant over the control

Fig. 27: Levels of total thiols in the brain mitochondria of FHF rats



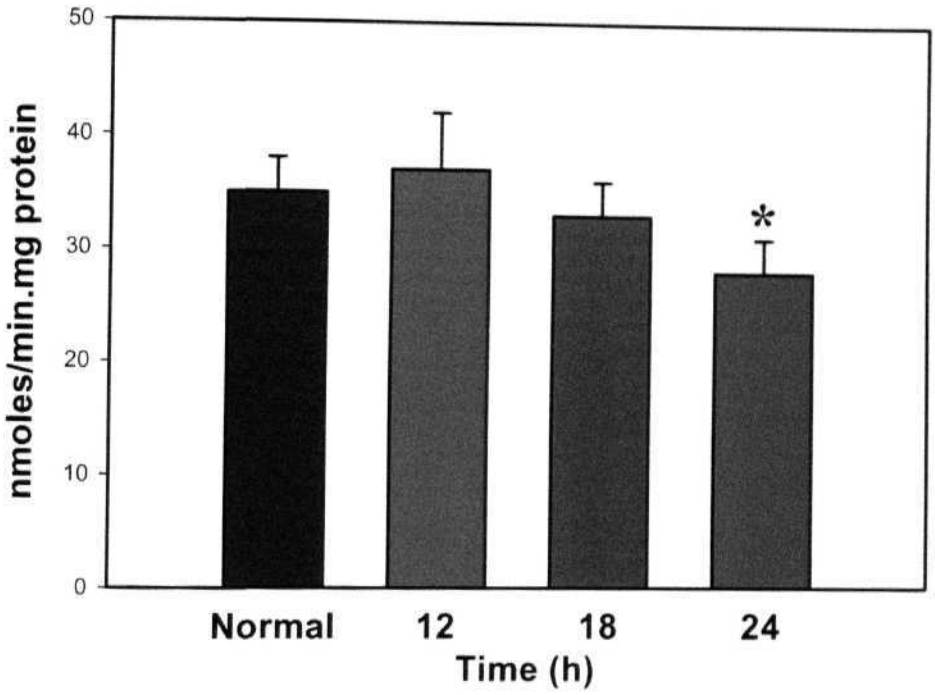
Changes in the total thiol content in the nonsynaptic mitochondria isolated from cerebral cortex of normal and thioacetamide-induced FHF rats at 12, 18 and 24h after the administration of the drug. All values are mean ± Standard deviation of six individual experiments done in duplicates.
* Statistically significant over the control.

Fig. 28: Effect of thioacetamide-induced FHF on glutathione peroxidase activity



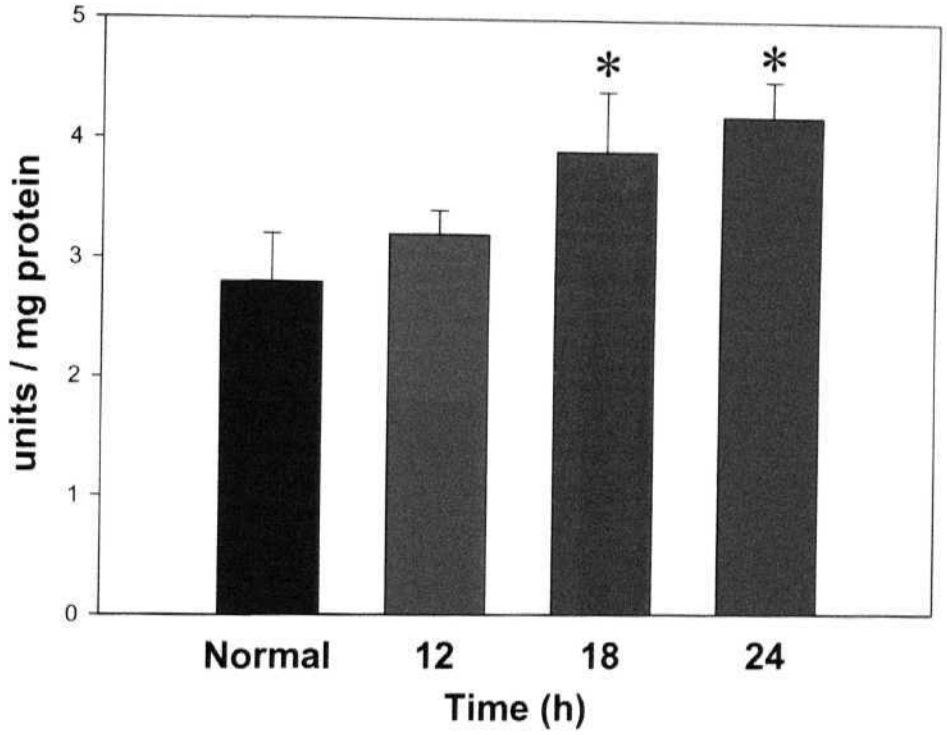
Changes in the activity of glutathione peroxidase in the nonsynaptic mitochondria isolated from cerebral cortex of normal and thioacetamide-induced FHF rats at 12, 18 and 24h after the administration of the drug. All values are mean ± Standard deviation of six individual experiments done in duplicates.
* Statistically significant over the control.

Fig. 29: Effect of thioacetamide-induced FHF on glutathione reductase activity



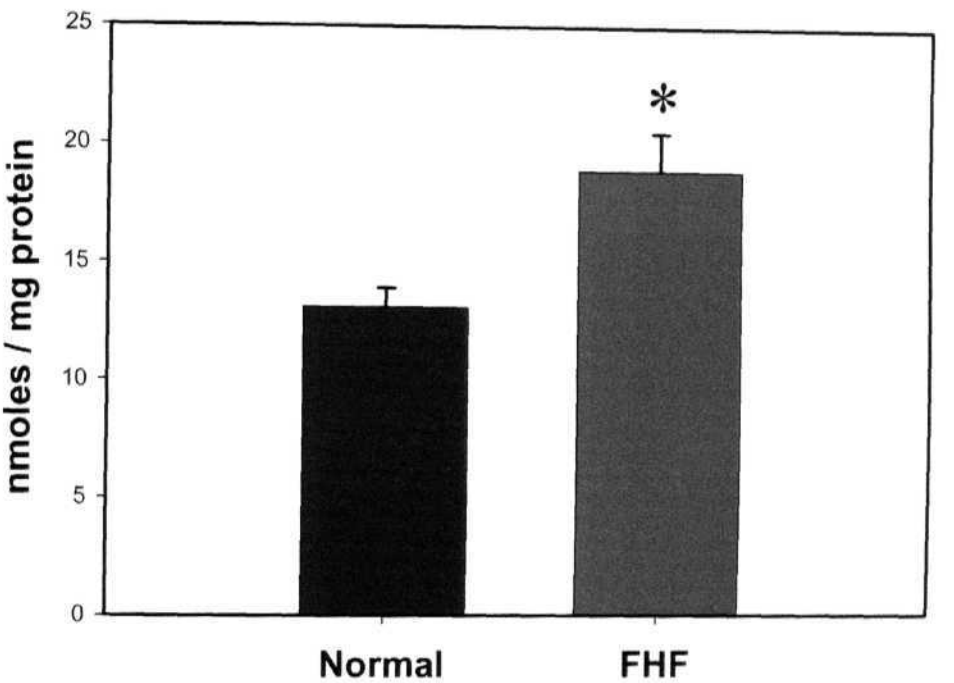
Changes in the activity of glutathione reductase in the nonsynaptic mitochondria isolated from cerebral cortex of normal and thioacetamide-induced FHF rats at 12, 18 and 24h after the administration of the drug. All values are mean ± Standard deviation of six individual experiments done in duplicates.
* Statistically significant over the control.

Fig. 30: Effect of thioacetamide-induced FHF on Mn-superoxide dismutase activity



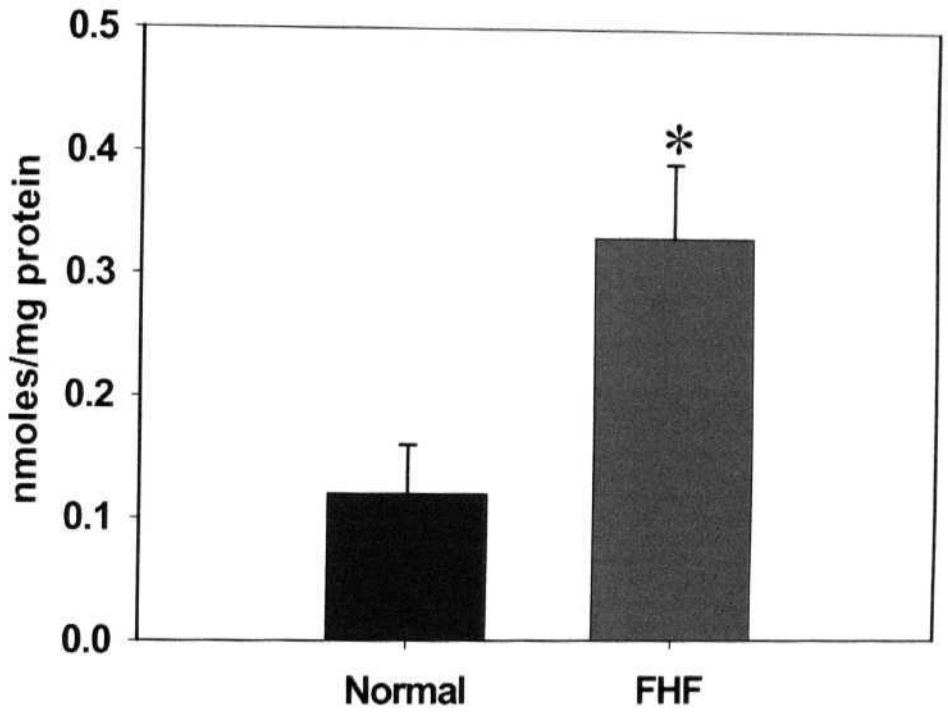
Changes in the activity of Mn-superoxide dismutase in the nonsynaptic mitochondria isolated from cerebral cortex of normal and thioacetamide-induced FHF rats at 12, 18 and 24h after the administration of the drug. All values are mean ± Standard deviation of six individual experiments done in duplicates.
* Statistically significant over the control.

Fig. 31: Changes in the content of GSH in nonsynaptic mitochondria of normal and thioacetamide-induced FHF rats



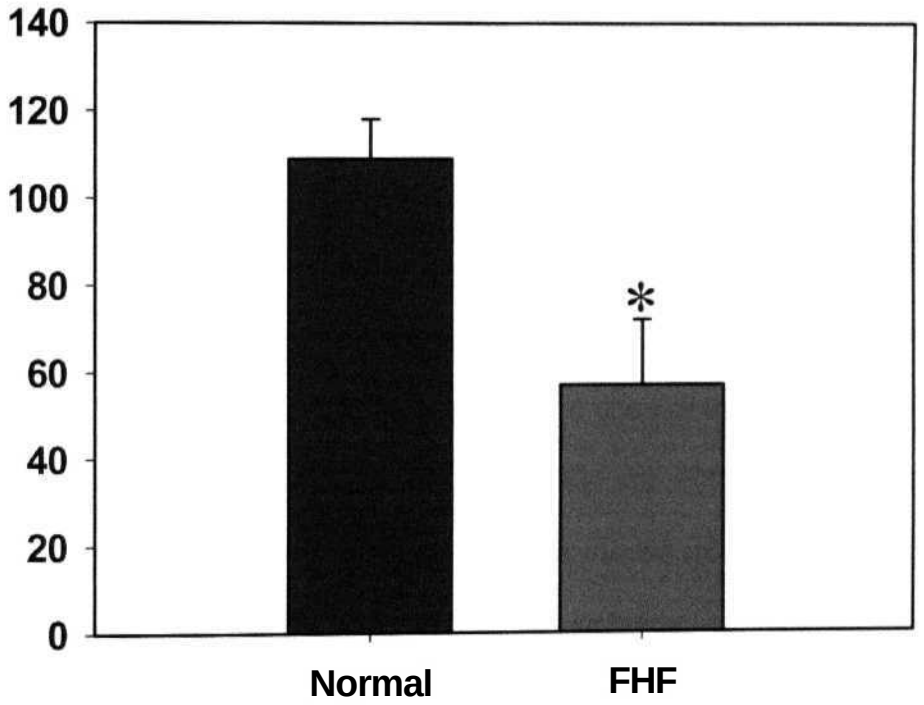
GSH content in the in the nonsynaptic mitochondria isolated from cerebral cortex of rats after 18 h after the administration of thioacetamide. Values are mean ± Standard deviation of six sets of experiments.
* Statistically significant over the control.

Fig. 32: Changes in the content of GSSG in normal and thioacetamide-induced FHF rats



GSSG content in the in the nonsynaptic mitochondria isolated from cerebral cortex of rats after 18 h after the administration of thioacetamide. Values are mean ± Standard deviation of six sets of experiments.
* Statistically significant over the control.

Fig. 33: Changes in the GSH/GSSG ratio of normal and thioacetamide-induced FHF rats



GSH/GSSG ratio was calculated from six sets of experiments and expressed as mean ± Standard deviation
* Statistically significant over the control.

Discussion

Fulminant hepatic failure (FHF) is a condition with sudden onset of necrosis of hepatocytes and degeneration of liver tissue without any established liver disease. FHF is associated with increased ammonia levels in blood and brain, which is supposed to be neurotoxic, ultimately leading to neuronal death. Evidences from previous studies suggest for mitochondrial dysfunctions under hyperammonemic conditions.

In the present investigation, a thioacetamide induced FHF rat model was developed and characterized. Results of the work that has been discussed in previous chapter clearly established the induction of fulminant hepatic failure by the administered dose of thioacetamide into the rats. Having established the rat model for FHF, attention was focused on to the involvement of the nonsynaptic mitochondria in the pathophysiology of the disease. The isolation of non synaptic mitochondria from brain specimens is complicated by the presence of myelin and synaptosomes which co sediment with mitochondria in normal differential centrifugation. In order to avoid the contamination of the mitochondria with the synaptosomes and with the vesicles formed by the sheared nerve endings during homogenization, metabolically active contamination-free nonsynaptic mitochondria were used in the present study. Non synaptic mitochondria from the cerebral cortex of adult Wistar rats were isolated by following the method of Cotman *et al.,* (1974) as described by Ratnakumari and Murthy (1989).

Pathological concentrations of ammonia are known to affect cerebral energy metabolism either by draining away the intermediates of TCA cycle for ammonia detoxification or by depleting cerebral energy reserves. Though there are reports on changes in intermediates, very little information is available on the activities of the enzymes involved in the energy metabolism such as reactions of the TCA cycle and electron transport chain complexes. Another possibility is that there may be uncoupling of oxidative phosphorylation leading to decreased energy production and mitochondrial damages. Hence the changes in the activities of the citric acid cycle enzymes were measured along with the alterations in the electron transport chain complexes in the non-synaptic mitochondria of thioacetamide induced FHF.

The activity of the pyruvate dehydrogenase (PDH) was lowered in the mitochondrial samples at 18 and 24 h of the thioacetamide treated rats. This decrease in the PDH activity would retard the channeling of pyruvate into TCA cycle. Citrate synthase activity which was measured by determining the formation of 5-thio-2-nitrobenzoate due to the coupling of CoA with DTNB at 412nm showed no change in the activity. Activity of citrate synthase *in vivo* depends on the availability of Acetyl Co A and oxaloacetic acid. The increase in α-ketoglutarate dehydrogenase activity observed in the present study would rapidly decarboxylate 2-oxoglutarate to succinate and thus pulls the isocitrate dehydrogenase reaction forward. Lack of change in

citrate synthase and elevation in α-ketoglutarate dehydrogenase would pull the isocitrate dehydrogenase reaction forward. Further, a decrease in the activity of succinate dehydrogenase (SDH) was observed in the drug administered FHF rats. Decrease in the activity of SDH might also alter the oxidation of succinate and thus the furtherance of the TCA cycle. An increase in α-ketoglutarate dehydrogenase activity and a decrease in SDH activity could lead to the accumulation of succinate, which leads to a subsequent decrease in rate of formation of malate and oxaloacetate. These changes in TCA cycle may have impact on synaptosomes also as synaptosomes also contain mitochondria.

Dysfunctions of mitochondria and electron transport chain (ETC) components (complex I, complex II, complex III and complex IV) have been implicated in a number of neurodegenerative disorders (Schapira *et al.,* 1996; Bowling *et al.,* 1995; Parker *et al.,* 1989; Mizuno *et al.,* 1995). Electron transport complexes have been reported to be the chief sites of free radical production in mitochondria (Turrens *et al.,* 1980; 1997; Chance *et al.,* 1979). The changes in the TCA cycle enzymes might lead to alterations in the electron transport chain complex.

The activities of complex I and III showed a significant decrease while complex II and IV remained unaltered in the thioacetamide induced hepatic failure rats. A decrease in the activity of complex I and III may lead to an elevation in rate of free radical production through ETC. Similar elevation in the generation of ROS as a result of the complex I and complex III was reported in sub mitochondrial particles

(Paradies *et al.,* 2001; Bayer *et al.,* 1992). Further, reports have also shown that the loss of these complex activities was prevented by the addition of exogenous free radical scavengers (Cardoso *et a*/., 1999; Paradies *et al.,* 2001). The inhibition of the complex III activity might be due to the loss of the mitochondrial membrane cardiolipin content as a result of the peroxidation of the membrane cardiolipin (Paradies *et al.,* 2000; 2001). This effect would lead to the leakage of electrons from the ETC and thus generating more of superoxide radicals and hence more free radical induced damage to the mitochondria. Inhibition of Complex III has been reported to lead the production of superoxide radicals (Cardoso *et al.,* 1999). The superoxide radicals will be dismutated to hydrogen peroxide, which in turn will react with superoxide radicals to produce more potent hydroxyl radicals. These hydroxyl radicals are even more devastating than the superoxide radicals. The enhanced production of these superoxide, H_2O_2 and hydroxyl radicals has been known to promote mitochondrial membrane alterations (Cardoso *et al.,* 1999). Moreover, the reaction of super oxide anion radicals increase the auto oxidation of the ubisemiquinones, leading to the enhanced release of the electrons from the ETC (Benzi *et al.,* 1991). Since brain demands high energy requirements, the inhibitions of the respiratory complex activities might have a pronounced effect on the functioning of the brain which could have an etiology in the pathophysiology of the disorder. Further complicating the situation are observations that indicate that free radicals can primarily affect complex I and III leading

to a decrease in their activities thus completing the vicious cycle. However, It remains to be established which of these two events precedes the other.

One of the possible mechanisms involved in the hyperammonemic or FHF conditions may be associated with the increased production of the reactive oxygen species (Dugan *et al.,* 1995; Piantadosi and Zhang, 1996). In such conditions studies have demonstrated the occurrence of depletions in the ATP levels in the brain (Hindfelt *et al.,* 1973; Kosenko et al., 1994; Kosenko *et al.,* 1993; McCandless *et al.,* 1981) which suggest that there could be changes in the mitochondrial respiration. For this purpose we studied the rates of different states of respiration in the mitochondria isolated from the TAA-induced FHF rats. The data indicate a decrease in the state 3 respiration of the mitochondria when pyruvate/malate was used as the substrate. This may be an indication for the lowered functional efficiency of the mitochondria in terms of the respiration. The decrease in the respiratory control ratio, when pyruvate/malate was used as the substrate might probably be due to the loss of integrity of the mitochondrial membrane. Similar findings were reported by Gracia *et al.,* (2003) and Kosenko *et al.,* (1997a) in the experimental conditions of ammonium acetate administered rats. Hindfelt and his group (Hindfelt *et al.,* 1977) have suggested that the defect in hyperammonemic conditions may be due to the inhibition of malate-aspartate shuttle which reduces the transport of redox equivalents to

the mitochondria. In support of this alterations in cytosolic and mitochondrial NADH/NAD ratios have been reported in brain in hyperammonemic states. The decrease in the P/O ratio however, was not statistically significant. But when it is considered over a large time period it might result in a significant decrease in the ATP synthesis.

The impaired functional efficiency of nonsynaptic mitochondria observed in the thioacetamide induced FHF might be resulting out of the structural damage to the mitochondria. Hence, further studies were under taken to check the structural integrity of the mitochondria in TAA administered rats. The studies of the photo refractory and TEM clearly indicated mitochondrial swelling during FHF.

Swelling of mitochondria leads to a decrease in the refractive index of the mitochondrial membrane which in turn could reduce the absorbance of light by mitochondria. This swelling in mitochondria may be due to the alterations in the structure of the membrane and loss of the integrity of the membrane. Loss of integrity of the membrane might lead to the damage to the selective permeability of mitochondrial inner membrane resulting in excess transfer of solutes and thus leading to the swelling of mitochondria.

Increased NMDA receptor activity in hyperammonemic conditions (Marcaida *et al.,* 1992; Hermenegildo *et al.,* 1996) is reported to be an effector of excitotoxic neuronal death (Beal, 1992). Elevated NMDA receptor activity results in increased Ca^{++} influx (Kosenko *et al.,* 1997; White *et al.,* 1996) which would hamper the ATP

synthesis and thus mitochondrial membrane potential. This might further result in mitochondrial electron transport chain alterations leading to enhanced production of free radicals (Choi, 1996; Nicotera *et al.,* 1997).

Reports from the literature suggest the possibility of oxidative stress in conditions of hepatic encephalopathy (Rao *et al.,* 1991; 1992, Kosenko *et al.,* 1997). Bai and group (Bai *et al.,* 2001) have reported collapse of mitochondrial membrane potential and permeability change as a consequence of oxidative stress in mitochondria of astrocytes exposed to pathophysiological concentrations of ammonia. Murthy and coworkers have further endorsed the production of ROS in a dose-dependent manner under these conditions (Murthy *et al.,* 2001). Taken together the aforesaid reports indicate that ammonia can induce excess production of free radicals, thereby affecting the mitochondrial integrity and thus mitochondrial function. However most of the studies mentioned above had used *in vitro* culture of astrocytes to depict the effects of hyperammonemia. Such studies do help in identifying the cellular site of action by avoiding either the complications arising out of inter organ and inter cellular interactions in *in vivo* studies or from other toxic/ protective factors produced in the body in response to the liver damage. The *in vivo* response of cells as opposed to *in vitro* responses is governed by a complex set of diverse interactions with neighboring cells and other extracellular components-their accessibility and concentration.

Results of the present study revealing a profound mitochondrial swelling, decrease in activities of complex I and III also have indicated and hinted that there could be enhanced production of free radicals in conditions of FHF, thus inducing oxidative stress in the diseased animals. Hence in the present study, generation of ROS and lipid peroxidation was undertaken in rat models of TAA induced FHF.

In the present study we have also evaluated the involvement of oxidative stress on non synaptic mitochondria isolated from cerebral cortex in conditions of hepatic encephalopathy. This was done by studying changes in lipid peroxidation, total thiols and various antioxidant enzymes after inducing FHF by administering thioacetamide. A better understanding of the role of mitochondria in generating the ROS as well as the consequences of oxidative stress will surely contribute to the existing knowledge on FHF disorder. Assays on SOD, GPx, GR, MDA, total thiols chosen in the present study will conveniently evaluate the role of oxidative stress, if any, in the pathophysiology of FHF. These studies resulted in an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the nonsynaptic mitochondrial lipid peroxidation. There was an increase in lipid peroxidation by 46% as early as 12h after thioacetamide administration. At 18h, it was further increased by 140%, and then by 180% at 24h after the administration of thioacetamide. The levels of total thiols, on the other hand, were decreased by 14% at 12h interval and 30% by 18h when compared with the controls. There was

no significant change in the activity of glutathione peroxidase at 12h after thioacetamide administration but decreased significantly by 14% and 24% at 18 and 24 h respectively. The activity of glutathione reductase also decreased by 17% over the controls, but only at 24 h. There was an increase of 39% and 46% in the activity of Mn-SOD at 18 and 24 h respectively after the administration of the thioacetamide.

Thus the studies on thioacetamide-induced FHF in rats reveal the induction of oxidative stress in nonsynaptic mitochondria as evidenced by the increased lipid peroxidation, decreased thiols and impaired antioxidant defenses. Oxidative stress is a condition in which the production of free radicals is far in excess of their rate of detoxification by endogenous mechanisms (Rao, 2002). Being a highly aerobic tissue accounting for 20% of total oxygen consumed by the body, brain is prone and vulnerable for oxidative stress. Free radicals are produced in the normal course of respiration and is estimated to be about 1-3% of the total oxygen consumed by the tissue (Kowaltowski *et al.,* 1990). Furthermore, brain is rich in polyunsaturated fatty acids (Halliwell, 1992) and possesses high content of iron in certain areas, which is supposed to promote free radical production. Added to this, brain has low levels of antioxidant enzymes, low repair mechanisms and non-replicative neuronal cells (Halliwell, 1992). All the aforesaid factors play a critical role in balancing the oxidative stress and the antioxidant defenses. Mitochondria are the major sites of production of free radicals especially by the respiratory electron transport chain

(Chance *et al.,* 1979). Under normal physiological conditions the free radicals produced are detoxified by a variety of endogenous free radical scavengers such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase etc. In the present condition of induced FHF, there is enhanced production of free radicals leading to lipid peroxidation coupled with impaired antioxidant defenses in nonsynaptic mitochondria, probably leading to their damage.

One of the major consequences of oxidative stress is lipid peroxidation. The free radicals oxidize the fatty acids present in the membranes leading to the production of lipid peroxides. Continuous increase in the formation of nonsynaptic mitochondrial malondialdehyde observed in the present study suggests that the peroxidation starts as early as 12 hours to reach a maximum at 24h time period. Lipid peroxidation is a chain reaction unless a check is imposed by antioxidant defenses. Impaired antioxidant defenses will increase the levels of peroxides, which eventually damage the membrane vesicles by altering the physicochemical properties of the membrane (Paradies *et al.,* 2001). Further the loss of the integrity of the mitochondrial membrane leads to mitochondrial dysfunctions, more precisely, respiration and oxidative phosphorylation (Masini *et al.,* 1985). Increase in the lipid peroxidation implies elevated production of free radicals *vis-à-vis* increased ROS to impose an inhibition on respiratory electron transport chain complexes (Masini *et al.,* 1985).

Mn-SOD is an important mitochondrial antioxidant enzyme and its activity in the present study was elevated in a time dependent manner after inducing FHF. Kosenko *et al.,* (2003) on the other hand, reported decreased Mn-SOD activity after the injection of ammonium acetate in rats. The apparent discrepancy in our results is not clear at the moment. Nevertheless, administration of ammonium acetate will create acute toxicity right away where as thioacetamide-induced FHF will be counter-acted by the system, which might have apparently reflected in the elevation of Mn-SOD activity. This observed increase in Mn-SOD activity in response to thioacetamide administration may be an attempt to detoxify the increased ROS and thus protect the tissue by dismutation of the $O^{2\cdot-}$ radicals. Mn-SOD is well known for its role in the primary cellular defense by protecting the cell from deleterious effects of oxygen free radicals (Fridovich, 1986). Overexpression of SOD results in the enhanced production of hydrogen peroxide that could result in lipid peroxidation especially if it is not reduced by peroxidases (Ceballos-picot *et al.,* 1991). A similar situation exists in the present study with enhanced SOD activity and decreased peroxidase activity levels in response to thioacetamide administration. Although an increase in SOD activity has a protective role, its increase with a simultaneous reduction in the activity of GPx is lethal to the tissue due to the accumulation of more hydroperoxides (Avraham *et al.,* 1998; Devi *et al.,* 1996). Similar observation of decreased GPx and GR activities was reported by Kosenko *et al.,* (2003) in

hyperammonemic states. The levels of total thiols indicate the redox state of the cell. The oxidation of thiols to disulfide (-S-S-), is a well-known sensitive indicator of oxidative stress (Sen *et al.,* 2000). GR is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH) and maintains a balance of the reduced glutathione levels. Decreased GR activity observed in the present study indicates the impaired redox cycling of GSH leading to oxidative stress in the brains of FHF animals.

Decreased enzymatic activities of glutathione peroxidase and glutathione reductase, and an elevated Mn-SOD activity observed in the present study might lead to elevated levels of H_2O_2 (Devi *et al.,* 1996; Dogru-Abbasoglu *et al.,* 2001). This could lead to oxidative stress in the mitochondria of FHF rats. Similar observations have been made by Dogru-Abbasoglu and his group (Dogru-Abbasoglu *et al.,* 2001) in the liver during thioacetamide induced hepatic failure. Oxidative stress has been shown to cause mitochondrial dysfunction (Paradies *et al.,* 2001) which is implicated in many neurological disorders (Heales *et al.,* 1999). This aspect, however, has to be further investigated.

TAA treatment of rats is characterized by marked elevation in serum and brain ammonia levels. The hyperammonemic state prevailing during TAA treatment may by itself lead to impairment of antioxidant enzyme functions as shown under *in vivo* conditions by the administration of ammonium acetate (Kosenko *et al.,* 1997b;

1999; 2003). Consistant with this Murthy et al (Murthy *et al.,* 2000) have shown the suppression of ammonia induced swelling in *in vitro* exposure of cells to antioxidant enzymes. Further studies of Staub *et al.,* (1994) and Norenberg *et al.,* (1991) also have shown that the free radicals may contribute to the cell swelling. However, the possible contribution of other factors that may be altered during TAA induced liver failure cannot be ruled out.

Glutathione is an abundant low molecular weight intracellular tripeptide, which is present in most of the cells. The presence of glutathione is required to maintain the normal function of the immune system. Glutathione is homeostatically controlled, both inside the cell and outside. Also glutathione is an important parameter that is implicated in the oxidative stress status of the cell. Furthermore, the cells produce many oxygen free radicals as a result of their normal functioning, resulting in a need for higher concentrations of antioxidants. Glutathione plays a crucial role in fulfilling this requirement. Glutathione also acts as a regulator for other antioxidants like vitamin C and E. It acts as a scavenger of reactive oxygen species, reactive nitrogen species, and potentially toxic oxidation products. It is also a substrate for the detoxifying enzymes like glutathione peroxidase, glutathione S-transferases and glutathione reductase. Glutathione exists in two forms, reduced (GSH) and oxidized (GSSG). The increase in the reduced form of glutathione in the nonsynaptic mitochondria of the thioacetamide treated rats may be due to the

increased activity of the γ-glutamyl-cysteine synthetase. Similar results were reported by Murthy *et al.,* (2000) in astrocyte cultures exposed to hyperammonemia. Generally a cell tries to counteract the imposed oxidative stress by stimulating the synthesis of reduced glutathione. The increase in reduced GSH observed in the present study may be an effort made by the cell to maintain the reduced state of the cell. Along with the increase in the total GSH content the oxidized form of glutathione (GSSG) was also enhanced. This increase in the GSSG makes the cell to accumulate more of the oxidized form of glutathione. Although an increase in both GSH and GSSG were observed, the magnitude of increase of the GSSG was much higher than the increase in GSH. This is further evident from the ratio of GSH/GSSG which is an indicator of oxidative stress. The decreased GSH/GSSG ratio in the present study indicates that the tissue is being pushed to a state where the oxidized form of glutathione gets accumulated. This decrease in the ratio of GSH/GSSG along with the increased lipid peroxidation and decreased antioxidant defenses suggest the possible induction of oxidative stress in the nonsynaptic mitochondria of the TAA-induced FHF in rats.

The foregoing studies thus indicate that the FHF induced in rats by thioacetamide administration results in impaired structural and functional organization of nonsynaptic mitochondria, mainly by the increased lipid peroxidation and impaired antioxidant defenses. This induced oxidative stress in nonsynaptic mitochondria might be due to

impaired energy metabolism as a result of diversion of TCA cycle intermediates and ATP towards the detoxification of accumulated ammonia in brain tissue of rats during FHF. The impaired structure and function of nonsynaptic mitochondria may account for the neuronal death associated with FHF condition. Further studies, however, are required to study the impaired function of brain in these conditions.

Conclusions

Conclusions

Fulminant hepatic failure (FHF) is a condition with sudden onset of necrosis of hepatocytes and degeneration of liver tissue without any established liver disease. FHF is associated with increased ammonia levels in blood and brain, which is supposed to be neurotoxic, ultimately leading to neuronal death. In hepatic failure surveillance study, a very poor survival rate (22%) was observed in the patients. Clinical outcome of the patients with FHF depends on the number of surviving hepatocytes, age, sex, etiology and the stage at which the patient is provided with medical help. There is no specific treatment or drugs to be administered to the patients with Fulminant Hepatic Failure and the outcome depends on the supportive intensive care and better patient management.

In order to have a clear understanding of the pathology in fulminant hepatic failure, blood, brain and liver samples are necessary. Though studies with human samples are highly helpful, this is not possible due to the ethical restrictions. Even if the material is available, we would find post mortem changes due to the lapse of time between the collection of sample and performance of the experiment. In addition, drugs administered in the course of treatment would also influence the biochemical parameters in the sample. Hence it would be difficult to infer whether the results obtained are really the experimental changes or due to the drugs used. These problems can be overcome by employing a suitable animal model that closely mimics the conditions observed in humans. For this purpose an animal model for **FHF** was generated in the present study by injecting thioacetamide.

An animal model for FHF was developed by the administration of two doses (300 mg/kg body weight) of thioacetamide into the rats intraperitoneally, at 24 h interval. These animals mimic very closely the human disease conditions evidenced by:

Increase in the levels of ammonia in the liver, serum and subsequently in the brain.

Elevation in the serum and liver urea levels

Decrease in the glucose levels in the liver and serum

Decrease in the protein content in liver and serum

Significant decrease in the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver with a corresponding increase in their activities in the serum

Significant prolongation in the prothrombin time

Progressive liver necrosis with impaired structural organization after thioacetamide administration

As shown above, TAA induced FHF is associated with increased ammonia levels in the brain, which is supposed to be neurotoxic and ultimately leading to neuronal death. However, the molecular mechanisms involved in FHF-induced neuronal death were not clearly delineated. It is possible that high concentrations of ammonia accumulated in the brain during FHF conditions might impair cerebral energy metabolism by draining away the TCA cycle intermediates for ammonia detoxification. Another possibility is that there may be uncoupling of oxidative phosphorylation leading to decreased energy production as a result of mitochondrial damages. Hence, in the present study, the changes in the activities of the citric acid cycle enzymes were measured along with the alterations in the electron transport chain complexes in the non-synaptic mitochondria of thioacetamide induced FHF. Also the effect of thioacetamide-induced FHF on mitochondrial respiration and their structural integrity has been studied. These studies resulted in the following changes:

Conclusions

- There was a decrease in the activities of pyruvate dehydrogenase and succinate dehydrogenase in the cerebral nonsynaptic mitochondria of the FHF induced rats.

- A significant increase in the activity of α-ketoglutarate dehydrogenase was observed in the nonsynaptic mitochondria of FHF rats.

- The activities of respiratory complex I and III of the mitochondrial electron transport chain showed a significant decrease.

- The activities of respiratory complex II and IV of the mitochondrial electron transport chain, however, remained unaltered.

- Photo refractory studies and transmission electron microscopic studies with the mitochondria clearly indicate a swelling in mitochondria during fulminant hepatic failure.

- No change was observed in state III and state IV mitochondrial respiration when succinate was used as the substrate.

- The respiratory control ratio was found to be unaltered when succinate was used as the respiratory substrate.

- A decrease in the state III respiration of the mitochondria was observed when pyruvate-malate was used as the respiratory substrate.

The respiratory control ratio was also found to be reduced in the mitochondria of the drug administered rats when pyruvate-malate were used as the substrates.

There was no significant change in the P/O ratios either with succinate or pyruvate-malate supported mitochondrial respiration.

The foregoing studies clearly indicate alterations in TCA cycle enzymes, leading to draining of the intermediates towards ammonia detoxifications in the nonsynaptic mitochondria of FHF rats. Also the activities of respiratory complexes of mitochondrial electron transport chain were significantly affected, possibly mediated by the impaired structural integrity of the mitochondria under these conditions. These studies suggest a possible oxidative damage to the mitochondria by either enhanced generation of free radicals or impaired antioxidant defenses during TAA-induced FHF. Hence, in the present study, lipid peroxidation was measured in the nonsynaptic mitochondria during TAA-induced FHF. Also the involvement of oxidative stress was evaluated by studying changes in antioxidant defenses. The following are the results of the above studies:

- In FHF rats, there was an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the lipid peroxidation in nonsynaptic mitochondria.

- The levels of total thiols in experimental animals, on the other hand, were decreased till 18 h when compared with the control. At 24 h, no further change was observed.

Conclusions

An increase in the reduced as well as oxidized form of glutathione was observed in the non synaptic mitochondria of FHF induced rats leading to overall increase in total GSH levels.

The ratio of GSH/GSSG, however, was decreased in the mitochondria of the rats affected with FHF suggesting higher degree of elevation of GSSG compared to that of GSH.

There was no significant change in the activity of glutathione peroxidase at 12 h interval but there was a statistically significant decrease in the latter time points of drug administered animals.

The activity of glutathione reductase was decreased at 24 h interval after the induction of FHF by the administration of thioacetamide.

An increase in the activity of Mn-SOD was observed at 18 and 24 h after the administration of the thioacetamide.

The foregoing studies thus clearly demonstrate the induction of oxidative stress in nonsynaptic mitochondria during TAA-induced FHF, as a result of enhanced lipid peroxidation and impaired antioxidant defenses. The results of the present thus suggest impaired structural and functional derangements in the cerebral mitochondria during thioacetamide induced FHF, possibly through enhanced lipid peroxidation and impaired antioxidant defenses. These cerebral mitochondrial dysfunctions may lead to neuronal death associated with FHF conditions.

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Fulminant hepatic failure induced oxidative stress in nonsynaptic mitochondria of cerebral cortex in rats

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Abstract

Fulminant hepatic failure (FHF) is a condition with sudden onset of necrosis of hepatocytes and degeneration of liver tissue without any established liver disease. FHF is associated with increased ammonia levels in blood and brain, which is supposed to be neurotoxic, ultimately leading to neuronal death. Evidences from previous studies suggest for mitochondrial dysfunctions under hyperammonemic conditions. In the present investigation, on thioacetamide-induced FHF rat models, studies were undertaken on cerebral nonsynaptic mitochondrial oxidative stress. The results of the present study reveal elevated lipid peroxidation along with reduced total thiol levels in the cerebral cortex mitochondria of experimental animals compared to saline treated control rats. In addition, the enzymatic activities of glutathione peroxidase and glutathione reductase were decreased, with an elevation in Mn-SOD activity. Overall, thioacetamide-induced FHF in rats enhanced the levels of lipid peroxidation coupled with impaired antioxidant defenses in the cerebral nonsynaptic mitochondria.

Keywords: Fulminant hepatic failure; Mitochondria; Thioacetamide; Oxidative stress

Fulminant or acute hepatic failure (FHF) is a condition with sudden onset of necrosis of hepatocytes and degeneration of liver tissue without any established liver disease. A wide range of neurological and neuropsychiatric alterations are seen in these patients and often death results due to cerebral edema [49].

Viral infections, over dosage of hepatotoxic drugs such as paracetamol (acetaminophen), halothane, tetracycline, valproic acid, anti-tuberculosis drugs, sulfonamides, diuretics, etc. [45,47] are reported to be the chief causes leading to FHF. Other known causes include food contaminants, such as aflatoxins, mushroom poisons, shellfish poisons, bacterial toxins and heavy metals, Wilson's disease and hepatic malignancy [9,20].

The precise pathophysiological mechanisms leading to the neurological alterations seen in this condition still remain an enigma. However studies in this area so far indicate that elevated levels of ammonia may be the chief culprit [18,1,40,10]. During hepatic inadequacy, as occurs in FHF, large quantities of ammonia in the portal blood escapes the detoxification process and enters systemic circulation. Thus, blood and tissue (brain) ammonia levels are elevated rapidly in FHF. Increased NMDA receptor activity in hyperammonemic conditions [35,26] is reported to be an effector of exitotoxic neuronal death [5]. Elevated NMDA receptor activity results in increased Ca^{2+} influx [30,57] which would hamper the ATP synthesis and thus mitochondrial membrane potential. This might further result in mitochondrial electron transport chain alterations leading to enhanced production of free radicals [17,39]. Several other hypotheses have also been proposed to explain the pathogenetic mechanisms involved in hyperammonemic conditions. These include disturbances in neurotransmitter functions, astrocytic abnormalities [40,41,4], increase in benzodiazepines and up regulation of their receptors

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[10,28], elevated serotonin synthesis [10,11], accumulation of endogenous opioids [11,12].

Reports from the literature suggest the possibility of oxidative stress in conditions of hepatic encephalopathy [51,52,30]. Bai et al. [4] have reported collapse of mitochondrial membrane potential and permeability change as a consequence of oxidative stress in mitochondria of astrocytes exposed to pathophysiological concentrations of ammonia. The same group has further endorsed the production of ROS in a dose-dependent manner under these conditions (38].

Taken together the aforesaid reports indicate that ammonia can induce excess production of free radicals, thereby affecting the mitochondrial function. However most of the above mentioned studies on this line had used in vitro culture of astrocytes to depict the effects of hyperammonemia. Such studies do help in identifying the cellular site of action by avoiding either the complications arising out of inter organ and inter cellular interactions in in vivo studies or from other toxic/protective factors produced in the body in response to the liver damage. The in vivo response of cells as opposed to in vitro responses is governed by a complex set of diverse interactions with neighboring cells and other extracellular components—their accessibility and concentration.

In the present study we have evaluated the involvement of oxidative stress on nonsynaptic mitochondria isolated from cerebral cortex in conditions of hepatic encephalopathy. This was done by studying changes in lipid peroxidation. total thiols and various antioxidant enzymes after inducing FHF by administering thioacetamide.

Thioacetamide, a selective hepatotoxin, is well known to induce hepatic failure [2]. Within a short period of time after the administration of the drug, thioacetamide is rapidly metabolized to acetamide and thioacetamide-*S*-oxide by the mixed function oxidases in the body [16]. Acetamide does not have liver necrotizing properties while thioacetamide-*S*-oxide is further metabolized by cytochrome P-450 monoxygenases to a sulfene, thioacetamide-*S*-dioxide. This thioacetamide-*S*-dioxide is a very highly reactive compound [27,48]. Its binding to the tissue macromolecules might induce hepatic necrosis [48j.

All the protocols followed for the use of the animal experimentation were approved by the institutional as well as the national ethical committee guidelines. Male rats of Wistar strain (weighing 300 g) were used in the present study. All the animals were housed four per cage at 25 ± 2 °C with 12 h day-night cycles in the animal house facility available at the University of Hyderabad. Food and water were provided to the animals ad libitum. Balanced pellet diet for the rats was supplied by Hindustan Lever Ltd.

Thioacetamide (300 mg/kg body weight) was dissolved in physiological saline and administered intraperitoneally for two days at 24 h interval. Animals were killed at different time periods (12, 18 and 24 h) after the administration of the second dose. Control rats received normal saline to serve as vehicle controls. All the rats were given a 25 ml/kg body weight of supportive therapy which consisted of 5% dextrose and 0.45% saline with 2()mEq./l of potassium chloride [43].

In order to assess the liver failure in thioacetamide treated rats, liver function tests were performed using specific biochemical markers. The methods for these tests were well standardized in our laboratory. The levels of ammonia in serum, liver and brain were determined according to the method of Ratnakumari and Murthy [53]. Serum and liver aminotransferases activities were measured as per the method of Bergmeyer and Brent [7]. Prothrombin time was determined by using standard commercial kit (obtained from Tulips, India). For studying the histopathology, the liver specimen was fixed in Bouin's fluid, embedded in paraffin wax, sliced the sections, stained the sections with haematoxylin and eosin and observed under light microscopy.

In order to avoid the contamination of the mitochondria with the synaptosomcs and with the vesicles formed by the sheared nerve endings during homogenization we used metabolically active contamination-free nonsynaptic mitochondria. Nonsynaptic mitochondria from the cerebral cortex of adult Wistar rats were isolated by following the method of Cotman [19] as described by Ratnakumari and Murthy [53]. The nonsynaptic mitochondria that were isolated from the brain were subjected to osmotic shock to disrupt them by treating with 10 mM phosphate buffer, pH 7.4 for 10 min and then they were subjected to three quick freeze-thaw cycles. The sample that was subjected to osmotic shock was centrifuged for 30 min at 1,00,000 x *g*. The supernatant thus obtained after this spin was collected for determining the activities of glutathione peroxidase, glutathione reductase and superoxide dismutase.

Activity of glutathione reductase (GR) (EC 1.6.4.2) was determined by following the procedure of Carl berg and Mannervik [13]. In brief, the reaction mixture (final volume 1 ml) consists of 0.2 M sodium phosphate buffer pH 7.0, 0.2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was initiated by the addition of mitochondrial protein sample and the oxidation of NADPH was recorded as decrease in absorbance at 340 nm for five minutes. Nonspecific oxidation of NADPH was measured in the absence of added GSSG and the enzyme activity was calculated using the molar extinction coefficient of NADPH. Activity of glutathione peroxidase (GPx) (EC 1.11.1.9) was measured according to the method described by Lawrence and Burk [34]. Both glutathione peroxidase and reductase activities were expressed as nmoles NADPH oxidized/min mg protein.

Total SOD activity was assessed according to the method described by Beauchamp and Fridovich [6] by measuring the degree of inhibition of the reduction of NTB in the presence of xanthine-xanthine oxidase system. Mn-SOD activity was calculated as the difference between the total activity(Cu,Zn-SOD and Mn-SOD) and the activity measured in the presence of Cu,Zn-SOD inhibitor cyanide. One unit of activity was defined as the amount of enzyme required to inhibit 50% NTB reduction rate.

Total thiols were estimated as per the method of Sedlak and Raymond [54]. Aliquots of 0.1 ml sample were mixed with 1.5 ml of 0.2 M Tris buffer, pH 8.2 and 0.1 ml of 0.01 M DTNB. The mixture was made up to 10ml with 7.9 ml of absolute methanol and it was incubated for 30 min. The mixture was then centrifuged at 3000 rpm for 15 min and the absorbance of the supernatant was read at 412 nm. The molar extinction coefficient of 13,100 was used to calculate total thiols.

Malondialdehyde, a by product of lipid peroxidation, was determined by the classical thiobarbituric acid assay of Ohkawa et al. [44] as described by Kosenko et al. [32]. In brief, the brain homogenates were prepared in 1.15% KC1. Mitochondria were prepared as described earlier and later suspended in 1.15% KC1. To 0.1 ml of the mitochondrial sample, 0.2 ml of 8.1% SDS, 1.5 ml of acetic acid (20%, pH 3.5) and 1.5 ml of 0.8%. thiobarbituric acid were added, and the final volume was made up to 4 ml. This total mixture was incubated at 90 °C for a period of one hour. The samples were then cooled and centrifuged at 1000 x *g* for 10 min at room temperature. The absorbance of the supernatant was measured at 535 nm with malondialdehyde (Sigma) as the standard.

Estimation of protein was done by the method of Bradford [8]. Mitochondrial isolation for the determination of the enzyme activities was done fresh everyday and used on the same day.

The animal model for fulminant hepatic failure was developed by administering thioacetamide (300 mg/kg body weight) intraperitoneally consecutively for two days. To confirm the occurrence of FHF, various liver function tests (Table 1) and histopathological studies were performed. The results of these tests revealed that liver function is completely impaired with a progressive necrosis of liver with time after thioacetamide administration (Fig. 1). The saline treated control rats, on the other hand, were found normal.

These established animal models were used to study the involvement of oxidative stress on nonsynaptic mitochondria in conditions of hepatic encephalopathy. In the experimental animals with induced FHF, there was an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the nonsynaptic mitochondrial lipid peroxidation (Table 2). There was an increase in lipid peroxidation by 46% as early as 12 h after thioacetamide administration. At 18 h, it further increased by 140%, and then by 180% at 24 h after the administration of thioacetamide. The levels of total thiols, on the other hand decreased by 14% at 12h interval and 30% by 18 h when compared with the controls (Table 2). At 24 h, no further change was observed. There was no significant change in the activity of glutathione peroxidase at 12 h interval but there was a statistically significant decrease of 14% and 24% at 18 and 24 h, respectively (Table 2). The activity of glutathione reductase also decreased by 17% over the controls, but only at 24 h (Table 2). There was an increase of 39% and 46% in the activity of Mn-SOD at 18 and 24 h after the administration of the thioacetamide, respectively (Table 2).

The present study, on ihioacetamide-induced FHF rats, reveals that nonsynaptic mitochondria may be subjected to oxidative stress as evidenced by the increased lipid peroxidation, decreased thiols and impaired antioxidant defenses. Oxidative stress is a condition in which the production of free radicals is far in excess of their rate of detoxification by endogenous mechanisms [50]. Being a highly aerobic tissue accounting for 20% of total oxygen consumed by the body, brain is prone and vulnerable for oxidative stress. Free radicals arc produced in the normal course of respiration and is estimated to be about 1-3% of the total oxygen consumed by the tissue [33). Furthermore, brain is rich in polyunsaturated fatty acids [24j and possesses high content of iron in certain areas, which is supposed to promote free radical production. Added to this, brain has low levels of antioxidant enzymes, low repair mechanisms and non-replicative neuronal cells [24]. All the aforesaid factors play a critical role in balancing the damaging effects and the antioxidanl defenses. Mitochondria are the major sites of production of free radicals especially by the respiratory electron transport chain [15]. Under normal physiological conditions the free radicals produced are detoxified by a variety of endogenous free radical scavengers such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, etc. In certain pathological conditions, however, the tissue or the cell will be subjected to a condition called oxidative stress, as the production of free radicals increase beyond the capacity of the endogenous protective mechanisms. A better understanding

Table 1
Effects of thioacetamide on serum and tissue biochemical parameters in thioacetamide-induced FHF rats

	Biochemical parameter	Control	12 h	18 h	24 h
Liver	Alanine amino transferase (μmol/g wet weight/h)	1532 ± 39	932 ± 45[a]	473 ± 27[a]	337 ± 41[a]
	Aspartate amino transferase (μmol/g wet weight/h)	2105 ± 67	1106 ± 32[a]	629 ± 46[a]	418 ± 53[a]
	Ammonia (μmol/g wet weight)	0.52 ± 0.07	0.83 ± 0.04[a]	2.31 ± 0.09[a]	4.27 ± 0.16[a]
Serum	Alanine amino transferase (μmol/ml/h)	4.23 ± 0.44	19.31 ± 0.57[a]	27.3 ± 0.36[a]	29.97 ± 0.85[a]
	Aspartate amino transferase (μmol/ml/h)	7.02 ± 0.20	13.36 ± 0.13[a]	16.16 ± 0.29[a]	20.7 ± 1.04[a]
	Prothrombin time (s)	15.5 ± 0.57	132 ± 23[a]	1092 ± 11[a]	1284 ± 4[a]
	Ammonia (μmol/ml)	0.165 ± 0.04	0.62 ± 0.1[a]	0.98 ± 0.07[a]	1.53 ± 0.18[a]
Brain	Ammonia (μmol/g wet weight)	0.31 ± 0.05	0.44 ± 0.03	0.58 ± 0.07[a]	0.71 ± 0.71[a]

Values are mean ± S.D. of six individual experiments done in duplicates.
[a] Statistically significant over the control ($P < 0.05$).

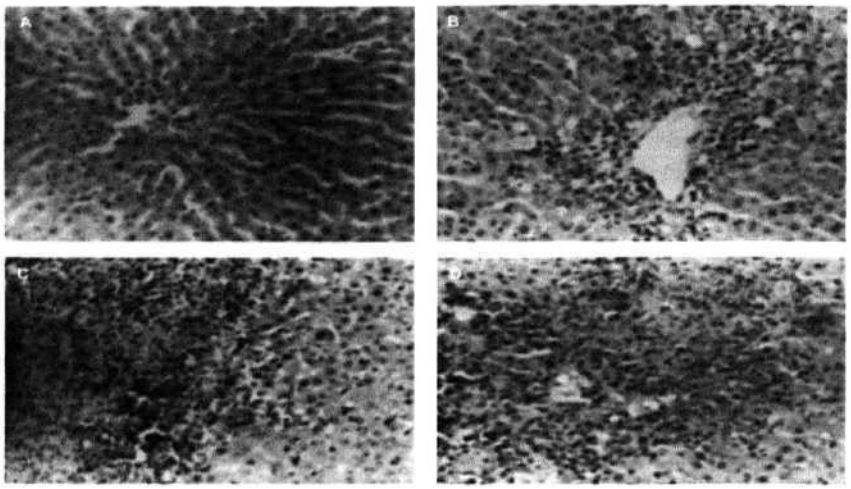

Fig. 1. Liver histology in thioacetamide-induced FHF. 40 x and 10× (A) Control, (B) 12 h, (C) 18 h, (D) 24h after the administration of the drug. Sections were stained with haematoxylin and eosin and observed under light microscope.

of the role of mitochondria in generating the ROS as well as the consequences of oxidative stress will surely contribute to the existing knowledge on FHF disorder. Assays on SOD, GPx, GR, MDA, total thiols chosen in the present study will conveniently evaluate the role of oxidative stress, if any, in the pathophysiology of FHF.

One of the major consequences of oxidative stress is lipid peroxidation. The free radicals oxidize the fatty acids present in the membranes leading to the production of lipid peroxides. Continuous increase in the formation of nonsynaptic mitochondrial malondialdehyde observed in the present study suggests that the peroxidation starts as early as 12 h to reach a maximum at 24 h time period. Lipid peroxidation is a chain reaction unless a check is imposed by antioxidant defenses. Impaired antioxidant defenses will increase the levels of peroxides, which eventually damage the membrane vesicles by altering the physicochemical properties of the membrane [46]. Further the loss of the integrity of the mitochondrial membrane leads to mitochondrial dysfunctions, more precisely, respiration and oxidative phosphorylation [36]. Increase in the lipid peroxidation implies elevated production of free radicals vis-a-vis increased ROS to impose an inhibition on respiratory electron transport chain complexes [36].

Mn-SOD is an important mitochondrial antioxidant enzyme and its activity in the present study was elevated in a time dependent manner after inducing FHF. Kosenko et al. [32] reported, on the other hand, decreased Mn-SOD activity after the injection of ammonium acetate. The apparent discrepancy in our results is not clear at the moment. Nevertheless, administration of ammonium acetate will create acute toxicity right away where as thioacetamide-induced FHF will be counter-acted by the system, which might have apparently reflected in the elevation of Mn-SOD activity. This observed increase in Mn-SOD activity in response to thioacetamide administration may be an attempt to detoxify the increased ROS and thus protect the tissue by dismutation of the $O^{2-\bullet}$ radicals. Mn-SOD is well known for its role in the primary cellular defense by protecting the cell from deleterious effects of oxygen free radicals [23]. Over expression of SOD results in the enhanced production of hydrogen peroxide that could result in lipid peroxidation especially if it is not reduced by peroxidases [14]. A similar situation exists in the present study with enhanced SOD activity and decreased peroxidase activity levels in response to thioacetamide administration. Although an increase in SOD activity has a protective role, its increase with a simultaneous reduction in the activity of GPx is lethal to the tissue due to the accumulation of more hydroperoxides [3,21]. Similar observation of decreased GPx and GR activities was reported by Kosenko et al. in hyperammonemic states [32]. The levels of total thiols indicate the

Table 2
Changes in the content of MDA. total thiols and alterations in the activities of various antioxidant enzymes in nonsynaptic mitochondria of thioacetamide-induced FHF rats

	Control	12 h	18 h	24 h
MDA (nmol MDA/mg protein)	6.08 ± 1.05	8.9 ± 0.65[a]	15.16 ± 0.70[a]	17.3 ± 0.92[a]
Total thiols (nmol/mg protein)	1.37 ± 0.2	1.175 ± 0.11	0.957 ± 0.12[a]	0.894 ± 0.1[a]
Mn-SOD (U/mg protein)	2.8 ± 0.4	3.2 ± 0.2	3.9 ± 0.6[a]	4.2 ± 0.3[a]
Glutathione peroxidase (nmol/min mg protein)	71 ± 3	76 ± 7	62 ± 5[a]	54 ± 9[a]
Glutathione reductase (nmol/min mg protein)	35 ± 5	37 ± 3	33 ± 4	28 ± 3[a]

Values are mean ± S.D. of six individual experiments done in duplicates.
[a] Statistically significant over the control ($P < 0.05$).

redox state of the cell. The oxidation of thiols to disulfide (-S-S-), is a well known sensitive indicator of oxidative stress [55]. GR is a flavoprotein that catalyzes the **NADPH**-dependent reduction of oxidized glutathione(GSSG) to glutathione (GSH) and maintains a balance of the reduced glutathione levels. Reduced GR activity observed in the present study indicates the impaired redox cycling of GSH leading to oxidative stress in the brains of FHF animals.

TAA treatment of rats is characterized by marked elevation in serum and brain ammonia levels (Table 1). The hyperammonemic state prevailing during TAA treatment may by itself lead to impairment of antioxidant enzyme functions as shown by Kosenko et al. [29,31,32] in vivo by administration of ammonium acetate. Consistant with this Murthy et al. [37] have showed in vitro exposure of cells to antioxidant enzymes resulted in the suppression of ammonia-induced swelling. Further, studies of Staub et al. [56] and Norenberg et al. [42] also have shown that the free radicals may contribute to the cell swelling. However, the possible contribution of other factors that may be altered during TAA-induced liver failure cannot be ruled out.

Decreased enzymatic activities of glutathione peroxidase and glutathione reductase, and an elevated Mn-SOD activity observed in the present study might lead to elevated levels of H_2O_2 [21,22]. This could lead to oxidative stress in the mitochondria of FHF rats. Similar observations have been made by Dogru-Abbasoglu et al. [22] in the liver during thioacetamide-induced hepatic failure. Oxidative stress has been shown to cause mitochondrial dysfunction [46] which is implicated in many neurological disorders [25]. This aspect, however, has to be further investigated.

Overall, our presenl study reports elevated lipid peroxidation coupled with impaired antioxidant defenses leading to reduced total thiols in the brains of thioacetamide induced FHF.

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