"BIOCHEMICAL ALTERATIONS IN TRYPTOPHAN METABOLISM LEADING TO OXIDATIVE STRESS IN BRAIN DURING FULMINANT HEPATIC FAILURE: ANTIOXIDANT PROTECTION BY C-PHYCOCYANIN"

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

BY

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HYDERABAD – 500 046, INDIA

I hereby declare that the work embodied in this thesis entitled **"Biochemical Alterations in Tryptophan metabolism leading to Oxidative stress in brain during Fulminant Hepatic Failure: Antioxidant protection by C-Phycocyanin"** has been carried out by me under the supervision of Late Prof.Ch.R.K.Murthy and Prof. P. Reddanna and this has not been submitted for any degree or diploma of any other university earlier.

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CERTIFICATE

This is to certify that **Mr. K.V. Sathya Sai Kumar** has carried out the research work embodied in the present thesis under Late Prof.Ch.R.K.Murthy and my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend his thesis **"Biochemical Alterations in Tryptophan metabolism leading to Oxidative stress in brain during Fulminant Hepatic Failure: Antioxidant protection by C-Phycocyanin"** for submission for the degree of Doctor of Philosophy of this University.

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Dedicated to my affectionate grandfather Sri K. Sitharamayya who suddenly passed away due to viral Hepatitis

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Sathya Sai Kumar K.V

ABBREVIATIONS

AA	: Amino acids
AAA	: Aromatic amino acids
AEF	: Astrocytic endfeet
AST	: Aspartate aminotransferase
ATP	: Adenosine triphosphate
AAT	: Alanine aminotransferase
AMPA	: Alpha-amino-3-hydroxy-5-methyl-4-isoxalepropionic acid
BBB	: Blood-brain barrier
BCAA	: Branched chain amino acids
BSA	: Bovine serum albumin
BV	: Blood vessel
^{0}C	: Degree Centigrade/ Degree Celsius
CC	: Cerebral cortex
CE	: Cerebellum
CNS	: Central nervous system
C-PC	: C-Phycocyanin
DAM	: Di acetyl monoxime
DCF	: Diclorofluoroscein
EB	: Evans blue
EDTA	: Ethylene Diamine Tetra Acetic acid
FHF	: Fulminant Hepatic Failure
g	: Gram
GABA	: Gamma amino butyric acid
GOD	: Glucose oxidase
GSH	: Glutathione reduced
GSSG	: Glutathione oxidized
GPx	: Glutathione peroxidase
GR	: Glutathione reductase
GST	: Glutathione S-transferase
h	: Hour(s)

HE	: Hepatic encephalopathy
3-HK	: 3-hydroxykynurenine
5- HIAA	: 5-hydroxyindole acetic acid
5-HT	: 5-hydroxy tryptamine (serotonin)
IDO	: Indoleamine 2,3 dioxygenase
ICC	: Immunohistochemistry
KP	: Kynurenine pathway
Da	: Dalton
DCP	: Diphenylcarbazide
SP	: Serotonin pathway
Kyn	: Kynurenine
Kyna	: Kynurenic acid
Kg	: Kilogram
KP	: Kynurenine pathway
MDA	: Malondialdehyde
mg	: Milligram
min	: Minutes
ml	: Milliliter
mM	: Millimolar
NAD	: Nicotinamide adenine dinucleotide
NADH	: Nicotinamide adenine dinucleotide reduced
NADPH	: Nicotinamide adenine dinucleotide phosphate reduced
NH_3	: Non ionic form of ammonia
NH_4^+	: Ionic form of Ammonia
NGF	: Nerve growth factor
nm	: Nanometers
NMDA	: N-methyl-D-aspartate
NO	: Nitric oxide
OPT	: O-pthalaldehyde
PBS	: Phosphate buffer saline
PM	: Pons Medulla

PB	: Phosphate buffer
PT	: Prothrombin time
QUIN	: Quinolinic acid
ROS	: Reacive oxygen species.
SOD	: Superoxide dismutase
TAA	: Thioacetamide
TEA	: Triethanolamine
TEM	: Transmission Electron Microscopy
Tris	: Tris-(Hydroxymethyl) aminoethane
Trp	: Tryptophan
Tyr	: Tyrosine
UV	: Ultraviolet
v/v	: Volume/volume
μM	: Micro molar
NEM	: N- Ethyl Maleimide

Contents

Introduction

1. 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 forms, Fulminant (acute) and Chronic respectively based on the type of liver dysfunction. Fulminant hepatic failure is a condition with a sudden onset of necrosis of hepatocytes and degeneration of liver without any previously established liver disease (Katelaris et al., 1989; Sherlock et al., 1971). This condition is usually associated with severe inflammatory necrotic liver damage and fatty degeneration of the liver and occurs rapidly (mostly within a week). Chronic HE (cirrhosis), on the other hand, is very slow in its progression and the symptoms develop very slowly and gradually. Although there are certain common aetiological factors, such as hyperammonemia, in the two forms of HE, there are distinct clinical courses and neuropathological endpoints between the two (Butterworth, 1992), thus making extrapolation of findings in one form to other difficult. The present study is mainly focused on the former (acute) type of Hepatic encephalopathy.

The chief causative factor for the acute hepatic encephalopathy was found to be viral infection. Infections related to viral hepatitis type B alone accounts for more than 74% in this category (Floersheim et al., 1975; Saunders et al., 1972). Contributions from non- A and non-B type of viral hepatitis accounts for 24%, while type A accounts for only 2% (Papas-Venegelu et al., 1984) in this

category. Ingestion of hepatotoxins is the next major cause of fulminant hepatic failure. These include indiscriminate usage of drugs such as paracetamol (acetaminophen) (Morton et al., 2000; Baudouin et al., 1995), tetracycline, valproic acid, halothane (Braude et al., 1981), sulphonamides, anti-tuberculosis drugs like isoniazid (INH), rifampicin (Plum and Hindfelt, 1976; Papas- Venegalu, 1984), anti convulsants such as phenytoin, carbamazene and phenobarbitol (Makin and Williams 1997; Seef et al., 1986), diuretics etc. Hepatotoxicity related to acetaminophen is the most frequent cause of FHF in the United Kingdom accounting for 60-70% of cases in recent times (Anand et al., 1997). This has also been reported to be the most prevalent cause of FHF from Denmark and United states (Larsen et al., 1995; Lee, 1993). Over dosage of these drugs especially paracetamol which most commonly acts as a hepatotoxin when used indiscriminately. Food contaminants such as mushroom poisons, aflatoxins, shellfish poisons, bacterial toxins and some heavy metals can also produce massive necrosis of hepatocytes causing FHF (Mullen et al., 1985; Hoyumpa et al., 1985; Papas-Venegelu et al., 1984; Braude et al., 1981; Delong et al., 1982; Burroughs et al., 1982).

A wide spectrum of neurological, neuropsychiatric and neuromuscular derangements are usually associated with the different stages of FHF. During the early stages, very subtle disturbances in sleep rhythms, personality and emotional changes are found. As the condition deteriorates, hypothermia, hyperventilation, confusion and drowsiness are seen (Jalan, 2003; Gayed, 1987; Margolis, 1979; Strauss et al., 1998). These changes lead to coma

and finally death (Kanamori et al., 1996; Dejong et al., 1993). Neuromuscular changes like asterixis, hyperflexia, unsustained clonus, deterioration of papillary, corneal, occulovestibular, occulocephalic and brain stem reflexes are also seen during different stages of FHF (Hoyumpa and Schenker, 1985). In addition, convulsions, cortical blindness, retarded speech, deterioration of electroencephalogram (EEG), motor plasticity and sixth nerve palsy are also observed in these patients (Hoyumpa and Schenker, 1985).

Statistical study of survival rates in FHF suggests a very poor survival rate (~22%) in human patients. The clinical outcome of these patients depends on the number of surviving hepatocytes, sex, age, etiology and the stage at which the medical help is given. As of now, there is no specific treatment or drugs that can be rendered to the patients of FHF. The outcome mainly depends upon the supportive intensive care and better patient management. However, even the management of FHF patients is less rewarding.

Patient management includes dietary restrictions, administration of lactulose, lactulose + neomycin sulfate, infusion of synthetic mixtures of amino acid or alpha keto acids, 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. However, the outcome is often not very satisfactory and the results are equivocal which is evident from the poor survival rate (~22%) of these patients (Riordan and Williams, 2000).

Multiplicity of the therapeutic practices and the uncertainties in their outcome are mainly due to the lack of clear understanding of the pathogenesis of the condition. This owes its reasons to various factors like: a) lack of appropriate animal model which closely simulates the human diseased condition; b) regional and cellular heterogeneity of the brain which renders studies more difficult, if not impossible and c) multiplicity of factors implicated in the etiology of cerebral dysfunction in HE.

1.2 Pathophysiological Mechanisms:

Intestine is the major site of ammonia production in the body (Weber and Veach, 1979). Bacterial action on dietary nitrogenous compounds (proteins, amino acids, nucleic acids etc.,) and intestinal metabolism of glutamine are responsible for the production of ammonia in the intestine. Ammonia, so formed is transported to the liver via portal blood and is converted to urea in periportal hepatocytes and to glutamine in perivenous hepatocytes (Haussinger et al., 1984). In conditions of hepatic insufficiency, ammonia is not sufficiently detoxified in liver and enters the systemic circulation. Elevation in blood and brain ammonia level is known to be neurotoxic (Schenker et al., 1974; Conn and Lieberthal, 1979; Cooper and Plum, 1987; Rukmini and Murthy, 1993; Butterworth et al., 1987; Felipo et al., 2002). Ammonia exists in two forms- unprotonated NH_3 and protonated NH_4^+ (ionic). Interconversion of these two forms is extremely rapid and depends on the pH of the medium. At physiological pH, about 97% of ammonia is in ionic form which is impermeable across the biological membrane.

When the pH is on the alkaline side, large amount of ammonia is present in the unprotonated form (NH_3) which is lipid soluble and highly permeable. This form is therefore considered to be potentially very toxic. However, in the present study, the term ammonia represents both the forms. In the central nervous system (CNS), ammonia is primarily metabolized/ detoxified to glutamine, as the complete urea cycle is absent. This action is carried out by glutamine synthetase (GS) (Martinez-Hernandez et al., 1977), in an adenosine triphosphate (ATP) consuming reaction that occurs predominantly in astrocytes (Norenberg and Martinez-Hernandez, 1979). This reaction involves the amidation of glutamate, which is mostly derived by uptake from the extracellular space. Glutamine is formed and then released from the astrocytes into the extracellular space, where it is taken up by neurons and converted to glutamate through the action of glutaminase, thereby completing the 'glutamate-glutamine cycle'. In liver failure, with marked elevation of the plasma and brain levels of ammonia, detoxification of the latter occurs via this pathway in brain leading to accumulation of glutamine.

1.2.1 Mechanisms of ammonia toxicity:

Though several mechanisms have been proposed to explain cerebral dysfunction in hyperammonemic states, most of the proposed mechanisms are equivocal (Cooper and Plum, 1987). Some investigators believe that excess ammonia levels in the brain exerts an adverse influence on cerebral energy metabolism (Bessman and Bessman,1955), either by depleting alpha ketoglutarate from citric acid cycle or by disrupting the transport of reducing

equivalents from cytoplasm to mitochondria through malate- aspartate shuttle (Siesjo, 1978). It has also been proposed that ammonia disrupts blood brain barrier (BBB) and affects the transport of several substances including ions resulting in cerebral edema (Ware et al., 1971). Another group of investigators believe that ammonia might be exerting its toxic effects by affecting the synthesis, release, re-uptake and post-synaptic action of neurotransmitters (Schaffer and Jones, 1982; Rao and Murthy, 1991; Rao et al., 1992). As glutamate and GABA are the chief excitatory and inhibitory neurotransmitters in mammalian brain (Danysz et al., 1995; Fonnum 1984; Ericinska et al., 1990), much of the attention was focused on these two neurotransmitters. Studies from our and other laboratories have shown that in hyperammonemic conditions, glutamate release is enhanced, while the reuptake (to terminate the neurotransmitter action) of glutamate by astrocytes is decreased in brain (Rao et al., 1991; 1992). 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 or 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 the GABA receptors (parallel and opposite to glutamate), loss of M_1 subtype of muscarinic acetyl choline receptors, receptors for dopamine, serotonin and opioid peptides have been reported in conditions of fulminant hepatic failure (Rao et al., 1991 & 1992; Van der Kloot, 1987; Fischer and Baldessarini, 1971). In addition the involvement of mercaptans (Zeive, 1980; Chen et al., 1970; Phear

et al., 1956) and short chain fatty acids (Butterworth, 2003; Zeive, 1980; Samson et al., 1956) have been implicated in the etiology of cerebral dysfunction in hepatic failure conditions.

1.2.2 Gamma amino butyric acid (GABA)

GABA, a neuroinhibitory substance produced in the gastrointestinal tract is thought to be involved in the pathogenesis of HE. GABA hypothesis was first proposed in HE by Schaffer and Jones (1982). During cirrhosis and FHF an increase in the GABA levels is observed in blood/circulation (Mullen et al., 1988; Maddison et al.,1987) which is probably due to the decreased metabolism of GABA in liver. 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 post synaptic potential. Administration of Benzodiazepines and barbiturates to patients with damaged liver or cirrhosis increases GABAergic tone and predisposes to depressed consciousness.

1.2.3 Benzodiazepines:

Another possible mechanism of enhancement of GABAergic neurotranmission relates to changes in the benzodiazepine receptor (BZR) or in its ligands. The BZR is part of the $GABA_A$ receptor complex and potentiates GABAergic inhibition when it is activated. An increase in the density of BZRs has

been reported in animal models (Schaffer et al., 1983; Baraldi et al.,1984; Leong et al., 1994) and in HE patients (Samson et al., 1987). These changes were contested by subsequent studies reporting no change in these receptors in HE, both in animal models (Pappas and Gordon, 1986) and humans (Basile et al., 1990 &1991a; Lal et al., 1987). Changes in the levels of ligands to the BZR have also been reported in HE (Mullen et al., 1988). This finding led to the hypothesis that the cause for the increased GABAergic tonus in HE might be due to an increase in the level of benzodiazepine like ligands.

1.2.4 Serum amino acids and false neurotransmitters:

In some patients and in animal models with HE, a decrease in the levels of branched-chain amino acids (BCAA; leucine, isoleucine, and valine) and an increase in the levels of Aromatic amino acids (AAA) (Tryptophan, Phenylalanine and Tyrosine) were observed (James et al., 1979). As these amino acids are transported across the blood-brain barrier by a single neutral amino acid transport carrier, it was postulated that there would be an increased influx of AAA into brain in the absence of functional liver. As a consequence, cerebral AAA content would exceed the K_m of the AAA hydroxylases (rate limiting reactions in the conversion of AAA into biogenic amines) and are directly decarboxylated to aromatic amines. Direct decarboxylation of AAA to aromatic amines lead to the formation of false neurotransmitters such as tyramine, tryptamine, β-phenylethylamine and octopamine (Basile et al., 1991b; James et al., 1979).

These false neurotransmitters bind to the pre- and post- 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 1994). Apart from depleting the pool of monoamines, false neurotransmitters can also suppress neurotransmitter synthesis by inhibiting the activity of tyrosine hydroxylase and dopamine β hydroxylase. They also act as partial agonists at the catecholamine receptors (Uprichard et al., 1977).

1.2.5 Other factors:

Role of other etiological factors, such as mercaptans (Phear et al., 1956; Chen et al., 1970; Zeive, 1980; Zeive and Brunner, 1985), short chain fatty acids (Samson et al., 1956; Zeive, 1983; 1985) etc., have not been worked out in detail. Increased blood levels of short chain fatty acids have been reported in the patients with hepatic insufficiency (Zeive, 1981). Reasons for such increase have not been clearly studied and moreover, many factors seem to be involved. In experimental animals, short chain free fatty acids produce reversible coma, but only at higher concentration (Hoyumpa and Schenker, 1985). It is unlikely therefore that these substances, by themselves, can be implicated in the etiology of human hepatic coma. However, synergistic action of the free fatty acids with other toxins such as ammonia can not be ruled out in the etiology of hepatic encephalopathy. Short chain fatty acids uncouple oxidative phosphorylation and have been shown to decrease the ATP and creatine phosphate content in the

brain stem regions, which is the seat for reticular activating system (Pappas-Venegelu, 1984; Cooper and Plum, 1987). Mercaptans such as methanethiol and dimethyldisulfide apparently formed as a result of gut bacterial metabolism can induce coma and convulsions by several mechanisms in HE patients including suppression of Na^+ K^+ ATPase activity. However, mercaptans concentration failed to correlate with the degree of HE.

1.3 Permeability of the blood- brain barrier.

In addition to the abnormal levels of toxic substances, changes in the blood-brain barrier (BBB) permeability have also been put forward as a contributing factor for pathogenesis of HE. Substances that accumulate in liver failure, such as ammonia, methyl octanoate, mercaptans, phenol or dehydrocholate (Zaki et al., 1984) may increase the permeability of the BBB through several different mechanisms. These mechanisms include a deleterious action on enzymes involved in the regulation of blood flow, changes in the function of glial transporter systems and an increase in the membrane fluidity (Goldstein, 1984). Basset et al., (1990) provided evidence that the permeability of the BBB increases for polar molecules (such as amino acids) in HE. On the other hand, other studies by Horowitz et al., (1983) suggest that the BBB permeability is non specific including both polar and non- polar substances. It should be emphasized that the findings suggesting increased permeability are more convincing regarding the cases of FHF, as there is little evidence of BBB changes in patients with chronic liver disease.

1.4 Tryptophan metabolism in brain – relavance to current study:

Tryptophan, not utilized for protein synthesis is metabolized in brain by two important pathways (Fig 1): The serotonin pathway leading to the formation of the neurotransmitter serotonin and the other namely, the kynurenine pathway leading to the formation of NAD^+ via formation of intermediates like 3-hydroxy kynurenine, 3-hydroxyanthranillic acid and quinolinic acid (QUIN).

1.4.1 Kynurenine pathway

There is good evidence that some metabolites derived from the kynurenine pathway (KP) are involved in the neurocytotoxicity associated with several brain diseases.(Heyes, 1996; Stone, 1993 & 2001a,b). The KP is a major route of L-tryptophan catabolism, resulting in the production of nicotinamide adenine dinucleotide and other neuro-active intermediates as shown in (Fig. 1) (Stone, 1993; Curzon, 1996; Bender and McCreanor, 1982; Botting, 1995; Moroni, 1999). These include kynurenine (KYN) (Lapin et al., 1982) kynurenic acid (KYNA), 3-hydroxykynurenine (3–HK) (Moroni, 1999; Eastman, 1989; Moroni et al., 1999), picolinic acid (Melillo et al., 1996) and quinolinic acid (Stone, 1993; Lapin et al., 1982; Schwarcz et al., 1983).

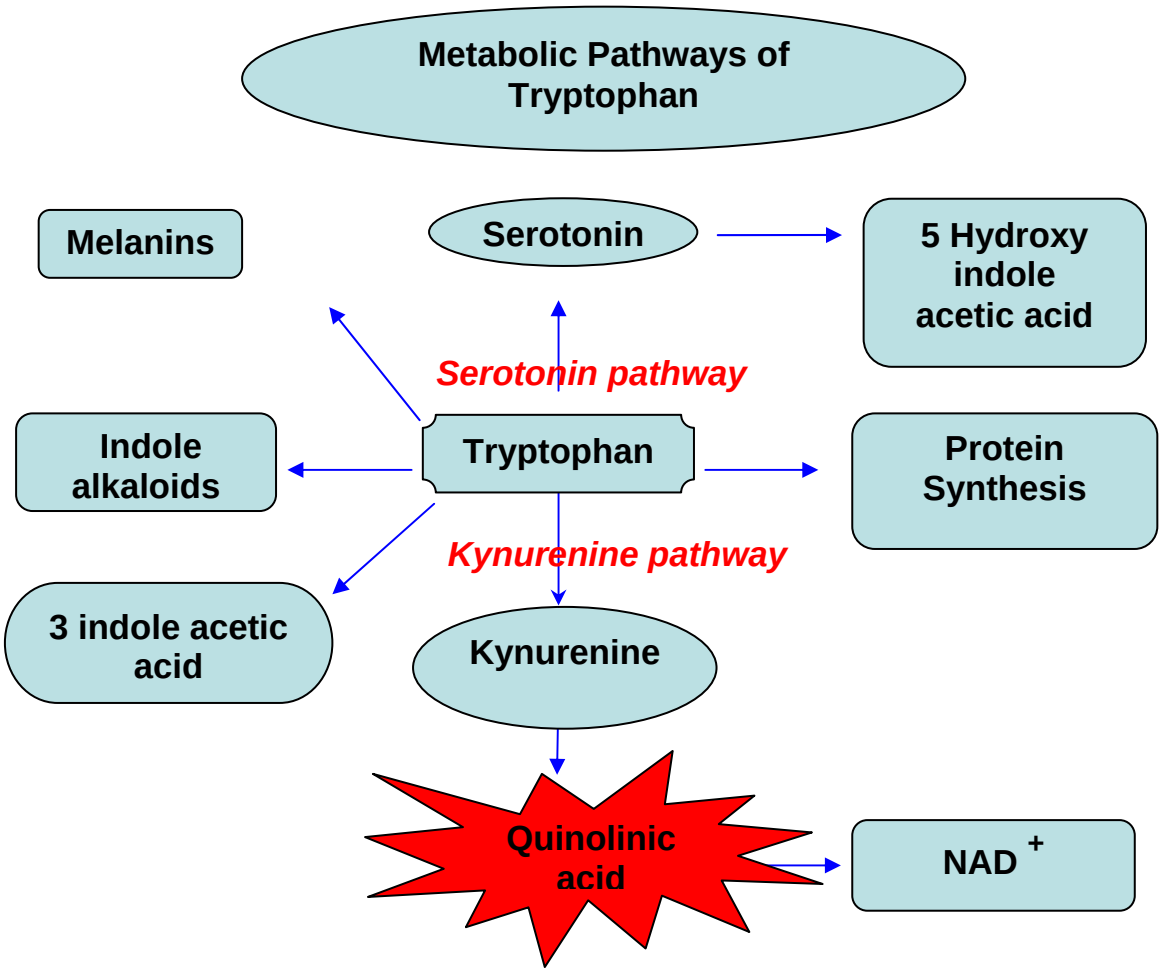


Fig: 1.1 Pathways of tryptophan metabolism

The KP also plays a role in certain physiological functions such as behavior, sleep, thermoregulation and pregnancy (Stone, 1993; Curzon, 1996). At present, it is not known whether the KP has any other major biological functions other than catabolism of Trp and synthesis of Nicotinamide. However, changes in the absolute or relative concentrations of the intermediates of kynurenine pathway have been implicated in a variety of central nervous system disorders such as AIDS-dementia, Parkinson's and Huntington's disease etc., raising the possibility

that interference with their actions or synthesis could lead to new forms of pharmacotherapy for these conditions.

Of the kynurenine pathway metabolites, the N-methyl D-aspartate (NMDA) receptor agonist and neurotoxin QUIN is perhaps the most important when present in excess to normal physiological levels. (Stone and Perkins, 1981; Stone, 2001b). It leads acutely to human neuronal death and chronically to dysfunction by at least four mechanisms. It can activate the NMDA receptor in pathophysiological concentrations (Kerr et al., 1995). Their activation leads to an influx of calcium into neurons, in turn triggering the activation of proteases, the formation of free radicals, the production of nitric oxide and other processes that can lead to neuronal death (Stone et al., 1987; Schwarcz et al., 1983; Kim and Choi, 1987). QUIN can inhibit post synaptic glutamate uptake and clearance from synaptic cleft leading to excessive micro-environment glutamate concentrations and neurotoxicity (Tavares et al., 2000). In addition to neuronal activation via NMDA receptors, quinolinic acid can induce mitochondrial dysfunction and the formation of reactive oxygen species inducing lipid peroxidation (Rios and Santamaria 1991; Behan et al., 1999; Santamaria et al., 2001a, b; Nakai et al., 1999). Lastly, QUIN can potentiate its own toxicity and that of other excitotoxins (*e.g*. NMDA and glutamate) in the context of energy depletion (Schurr and Rigor 1993; Bordelon et al., 1997). Chronic exposure to pathophysiologically concentrations of QUIN causes ultrastructural changes in cultured human neurons (Kerr et al., 1998). Another product of the KP pathway, Kynurenic acid, is an antagonist of all ionotropic glutamate receptors including

those for the glycine site of the NMDA (Stone, 1993), alpha-amino-3-hydroxy-5-methyl-4-isoxalepropionic acid (AMPA), and kainic acid receptors (Perkins and Stone, 1982) and thus can antagonize some of the effects of QUIN and other excitotoxins (Taber et al., 1996; Foster et al., 1984; Kerr et al., 1997; Boegman et al.,1990). However, it is not present in sufficient concentrations and the amounts of QUIN in the CSF, brain and systemic circulation always exceed KYNA by up to 15-folds (Heyes, 1993; Heyes et al., 1992; Freese et al., 1990). Kynurenine is another product which can cause a marked increase in expression of nerve growth factor (NGF) transcripts in astrocytes. KYN can be further metabolized to 3-hydroxykynurenine, which can lead to cell death by the generation of free radicals but only in the presence of another neurotoxin, especially QUIN; 3-HK alone does not cause neural damage presumably because the normal brain has efficient free radical scavengers to neutralize its effects (Dong-Ruy et al., 1998; Nakagami et al., 1996). 3-HK is known to be able to generate highly reactive free radicals which can synergize with relatively low doses of QUIN to potentiate its excitotoxicity (Guideti and Schwarcz, 1999). However, the cellular location of the KP is only partly understood. It is found in monocytic lineage cells, including macrophages, microglia (Espey et al., 1997, Heyes et al., 1992; Smith et al., 2001) and partly in astrocytes (Guillemin et al., 2001). The same group have also shown that astrocytes have a critical KP enzyme missing for the production of quinolinic acid and under physiological conditions, astrocytes do not produce quinolinic acid. Under normal physiological conditions small amounts of kynurenine is converted to Kynurenic acid, a

neuroprotectant. However, under pathological conditions where the astrocyte is stimulated, excess KYN is produced which can be taken up by monocytic lineage cells and processed through KP to yield neurotoxic concentrations of QUIN. As noted previously, the KP is switched on when microglia and astrocytes are activated. However, the degree of KP activation varies between individuals for unexplained reasons which renders some more susceptible to neurological disability than others. Recently it was reported that 3- hydroxyanthranillic acid, a good free radical generator is also capable of producing neuronal damage (Okuda *et al.,* 1996;1998).

1.4.2 Serotonin Pathway:

Approximately 1-2% of serotonin is contained in the central nervous system, where it is present in serotonergic neurons. Since serotonin does not penetrate the blood-brain barrier, neuronal serotonin is synthesized locally in the brain from the precursor amino acid tryptophan. Though it is only a minor pathway, the formation of serotonin is important because of its role as a neurotransmitter, neuromodulator and neuromediator. As a neurotransmitter, serotonin is involved in the regulation of feeding behavior, body weight, the sleep-wake cycle, aggression, impulsitivity, anxiety and depression (Cooper et al., 2003; Jacobs and Fornal, 1999). In addition to behavioral regulation, serotonin has been implicated in the pathological conditions like migraine, schizophrenia, obesity, depression and obsessive-compulsive disorders.(Cooper et al., 2003; Jacobs and Fornal, 1999). Changes in several neurotransmitter

systems have also been implicated in HE. Because of the impairment in amino acid metabolism induced by liver failure, the most investigated systems have been the amino acid and biogenic amine systems. Cerebral levels of serotonin were reported to be increased and the number of serotonin receptors on neurons was found to be decreased in HE and this could contribute to the neural inhibition in HE.

1.5 Oxidative stress:

Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue or organ caused by the reactive oxygen species (ROS). The steady state formation of pro-oxidants (free radicals) is normally balanced by a similar rate of consumption of antioxidants. Oxidative stress results from the imbalance between formation and neutralization of pro-oxidants. A complex natural antioxidant system exists in the biological systems, which is responsible for prevention of damage by pro-oxidants. Impaired endogenous antioxidant system leads to the accumulation of free radicals, which not only induces the process of lipid peroxidation but also plays a central role in neurodegeneration.

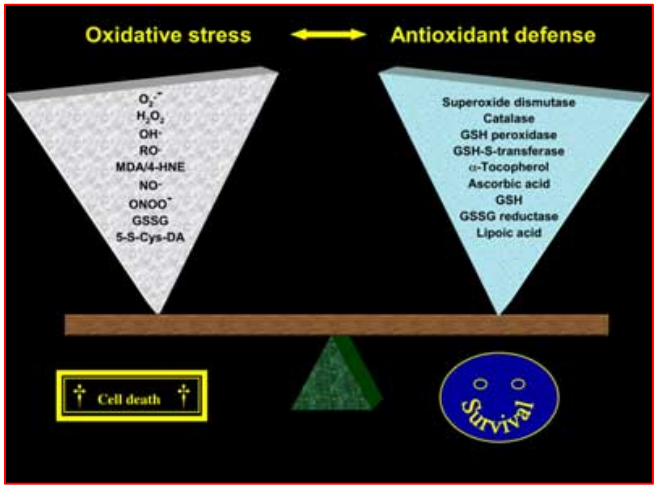


Fig: 1.2 Oxidants and antioxidants in biological systems maintaining a balance between cell survival and cell damage.

Free radicals are highly reactive molecules or chemical species capable of independent existence. They have one or more unpaired electrons with unpaired spin in their outer orbital, thereby possess increased reactivity with other molecules. In this process, they oxidize lipids in membranes, amino acids in proteins and carbohydrates and damage nucleic acids. Due to the prevalence of oxygen in biological systems oxygen centered radicals are called as “reactive oxygen species” (ROS). Generation of ROS is an integral feature of normal cellular function like mitochondrial respiratory chain, phagocytosis, arachidonic acid metabolism, ovulation and fertilization. Their production, however, multiplies several folds during pathological conditions. The most important ROS are the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), alkoxy radical ($RO^{\cdot}$), peroxyl ($ROO^{\cdot}$), hydroxyl radicals ($^{\cdot}OH$) and hypochlorous acid (HOCl). Other non-oxygen species existing as reactive nitrogen species (RNS), such as nitric oxide ($NO^{\cdot}$) and peroxynitrite also have important bioreactivity.

1.5.1 Oxidative stress in the nervous system:

The nervous system – including the brain, spinal cord and peripheral nerves – is rich in both unsaturated fatty acids and iron. The high lipid content of nervous tissue, coupled with its high aerobic metabolic activity, makes it particularly susceptible to oxidative damage. The high level of iron may be essential, particularly during brain development, but its presence is dangerous as brain cells may release iron ions during injury, which can lead to oxidative stress via the iron-catalyzed formation of ROS. In addition, those brain regions that are rich in catecholamines are more vulnerable to free radical generation. The catecholamine adrenaline, nor-adrenaline, and dopamine can spontaneously breakdown (auto-oxidize) to free radicals, or can be metabolized to yield free radicals by action of endogenous enzymes such as MAO (monoamine oxidase).

Oxidative stress has been implicated in most of the neurodegenerative diseases like Parkinson's disease, Huntington chorea and Aids-Dementia (Emerit et al., 2004). Tryptophan metabolites in brain seem to increase in all these pathological conditions thereby suggesting a link between these two. A number of studies indicated that QUIN induces oxidative stress in brain tissue *in vitro* and *in vivo* (Rios and Santamaria, 1991; Santamaria and Rios, 1993; Rodriguez-Martinez et al., 2000; Cabrera et al., 2000). Quinolinic acid is also known to be an endogenous glutamate agonist with a relative selectivity for the NMDA receptor causing neuronal death *in vitro* and *in vivo* (Moroni et al., 1986; Stone, 1993; Santamaria and Rios, 1993; Chiaurugi et al., 2001). Excitotoxicity produced by

sustained activation of NMDA receptors by quinolinic acid was associated with increased cytosolic Ca^{2+} concentration, ATP and gamma-aminobutyric acid depletion and specific GABAergic and cholinergic neuronal death (Foster et al., 1984; Schwarcz et al., 1984; Stone 1993). Recently it has been shown that quinolinic acid can also decrease the antioxidant enzyme activities and increase lipid peroxidation probably as a result of free radical generation (Leipnitz et al., 2005). This has severe consequences as it could lead to alterations in membrane fluidity, receptor function and ion permeability.

Further, increased lipid peroxidation was observed in hyperammonemic rats. Hyperammonemic states lead to NMDA activation and increased Ca^{2+} influx which could in turn activate various pathways involved in free radical generation in brain namely, iNOS and uncoupling of mitochondrial electron transport chain (Kosenko *et al.,* 1998 & 2003).

1.5.2 Antioxidant defenses:

Free radicals that are produced under physiological conditions are detoxified by a variety of antioxidant processes. The antioxidant defenses include both enzymatic and non-enzymatic ways. The non –enzymatic anti-oxidant defense includes, water soluble compounds such as ascorbate, glutathione, the lipid soluble membrane bound antioxidants- tocopherol and ubiquinol/ubiquinone. The enzymatic antioxidant defense includes super-oxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) and systems that prevent formation of

metabolism of pro-oxidants such as NADPH ubiquinone reductase and glucose6-phosphate dehydrogenase (Kerhrer and Lund, 1994). These defense mechanisms are normally able to keep reactive oxygen species to a level compatible with metabolic processes and cellular functions. However, in pathological conditions, reactive oxygen species escape the control of defense mechanisms reaching levels that may endanger the functional and structural integrity of the cell and, therefore bring reversible or irreversible damage. In view of the substantial oxidative stress observed in most neurodegenerative diseases, it is quite likely that antioxidants might protect the tissues from oxidative damage. Hence in the present study, the protective effect of C-Phycocyanin was evaluated.

1.6 C-Phycocyanin:

C-Phycocyanin (C-PC) is a copper containing protein isolated from ***Spirulina platensis.*** *Spirulina platensis* (formerly also called as *Arthrospira platensis)* is a non- nitrogen fixing , unicellular filamentous blue green algae with over 30 years long history of safe human consumption. C-PC is extensively used as a food colorant and in cosmetics because of its blue colour and its strong fluorescence in the visible region. It is also non-carcinogenic. Spirulina is gaining attention as a nutraceutical and as a source of potential pharmaceuticals. However, most of its pharmacological properties are not known except for a few. *Spirulina* with 62% protein content is the world's richest source of Vitamin B-12. It

is also rich in beta-carotene and other caretenoids, Vitamin E and minerals (e.g. Manganese, Copper, Iron, Zinc, and Selenium). The biological and pharmacological properties of Spirulina were mainly attributed to calcium-spirulan and C- Phycocyanin. C-PC consists of alpha and beta subunit polypeptides to which one or more linear tetrapyrrole chromophores are covalently attached. C-PC has significant antioxidant and radical scavenging properties (Romay et al., 1998; Vadiraja and Madyastha, 2000). C-PC has also been used in other neurodegenerative diseases such as Alzheimer's and Parkinson's (Rimbau et al., 1999). Studies from our laboratory, have also shown that C-PC selectively inhibits cyclooxygenase-2 (COX-2), an inducible isoform that is upregulated during inflammation and cancer, and induces apoptosis in mouse macrophage cells (Reddy et al, 2000;2003). The present findings clearly suggest that oxidative stress plays an important role in HE. Hence, in the present study an attempt is made to investigate the efficacy of C-Phycocyanin, a hepatoprotective biliprotein isolated from *Spirulina platensis*, in protecting the brain from HE.

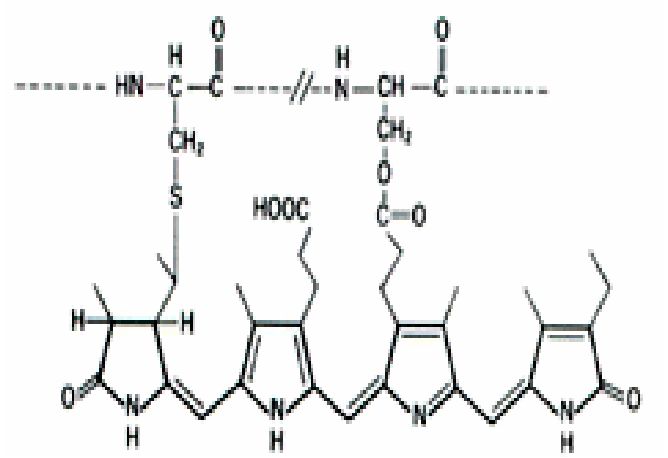


Fig: 1.3 Structure of C-Phycocyanin.

1.7 Rationale of the present study:

HE is characterized by changes in mood, confusion and alterations in sleep patterns. Many of these functions involve neurotransmitters derived from tryptophan and its metabolites. Further many of the neurodegenerative diseases like Huntington's chorea, Parkinson's and Aids-Dementia are characterized by changes in cerebral tryptophan metabolism. Some of the tryptophan metabolites are also potent free radical generators. Hence, in the current work, it is aimed to study these tryptophan metabolites and the oxidative stress in brain of rats induced with HE. Another objective of the current study would be to assess the protective effect of C-Phycocyanin, a known hepatoprotective agent and a strong antioxidant in conditions of HE.

1.5.1 Scope and Objectives:

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 spectrum of neurological, neuropsychiatric and neuromuscular changes is seen in conditions of fulminant hepatic failure. Much of the efforts have been on the development of a vaccine for viral infections. Though this may prove to be an effective measure for the prevention of this disease, it is not of much help since the cost of the vaccine, as of today, is out of reach of the rural population and there is no effective measure to prevent the clinical course once the disease process sets in. Further, vaccination is not

helpful in cases of ingestion of hepatotoxins. As mentioned earlier, survival of the patient depends on the management rather than on therapy. This becomes a problem in rural areas where the patient is admitted at very advanced stages and the patient management techniques are rudimentary. Hence, it becomes imperative to develop rational and effective therapeutic measures, which are in the reach of the rural population. Development of an animal model for FHF would aid in carrying out temporal and regional studies on the alterations in the cerebral functions and the mechanisms responsible for these changes, which is not possible in human samples. In addition, pilot studies on drug treatment can also be successfully carried out in an animal model.

The present study, as mentioned, is an effort to develop a unified hypothesis for the pathophysiology of cerebral dysfunction in fulminant hepatic failure and to find some protective agents which can ameliorate the toxicity of the hepatotoxin TAA.

Specific objectives:

- To establish an animal model for fulminant hepatic failure.
- To analyze “FHF-induced encephalopathy” in terms of :

a) Amino acid profiles in brain and serum

b) Histological changes in brain

c) Blood brain barrier changes

d) Metabolism of tryptophan in brain and

e) Oxidative stress

- To study the protective effects of C-Phycocyanin on “FHF-induced encephalopathy”.

Material And Methods

2. MATERIAL AND METHODS

2.1 Material

All amino acid standards were purchased from Fluka and tryptophan metabolites from Sigma Chemical Company (St Louis, MO, USA). The glucose, bilirubin and albumin kits were purchased from Glaxo, India;Ltd. Quinolinic monoclonal antibody was a generous gift from Gilles Guillemin, Centre for Immunology, St.Vincents Hospital, Sydney, Australia. Cu-Cd alloy was a kind gift from Dr.K.V.H.Sastry, Indian Avian Research Institute, U.P, India. C-Phycocyanin was a kind gift from Prof.K.Madyastha, IISc, Bangalore. Antibody against HNE was purchased from Alpha Diagnostic International (San Antonio, TX, USA). All HPLC solvents were obtained from SRL, India. All other chemicals not mentioned here, are procured from local companies and were of high purity grade.

2.2 Experimental Animals

Male rats (~300g) of Wistar strain were used in the present study. The animals were kept in cages (4 per cage) at 25 $\pm$ 2^{o}C with 12h day-night cycles in the animal house facility available at the University of Hyderabad. Animals had free access to water and food (Balanced pellet diet from Hindustan Lever Ltd.,). Permission was obtained from IAEC and CPCSEA for sacrificing the rats.

2.3 Drug administration

Thioacetamide (TAA, 300mg/kg body weight) dissolved in physiological saline was administered intraperitoneally for 2 days at a 24 h interval (Reddy et al., 2004). Animals were killed at different time periods (0,6,12,18,24&36h) after the administration of the second dose. Food and water were provided to the animals *ad libitum.* Control rats received normal saline served 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 Drug + C-PC Treatment

Thioacetamide (TAA, 300mg/kg body weight) + C-Phycocyanin (50 mg/kg body weight) dissolved in physiological saline was administered intraperitoneally for 2 days at a 24h interval. Animals were killed 18h 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).

2.5 Sample collection

Blood from normal and thioacetamide induced rats at specific intervals of time (0,6,12,18,24&36h) was drawn with a syringe by cardiac puncture and

transferred into clean dry centrifuge tubes. It was allowed to stand for 30 minutes at room temperature without any disturbance and the blood was allowed to clot. Serum was separated from the clotted blood by centrifugation at 5,000 rpm at 4^0C. This was used for the estimation of glucose, urea, proteins and enzymes such as aspartate and alanine amino transferases.

2.5.1 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^oC. 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 4^0C to remove precipitated potassium perchlorate. The supernatant was used for the estimation of ammonia.

2.5.2 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 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 4^0C. The supernatant thus obtained was neutralized as described for serum and used for the assay of ammonia.

2.5.3 Preparation of liver sample for biochemical estimations

Liver was excised from the control and thioacetamide treated rats respectively 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 estimation of enzyme activities and protein content.

2.6 Biochemical Characterization

2.6.1 Ammonia Estimation

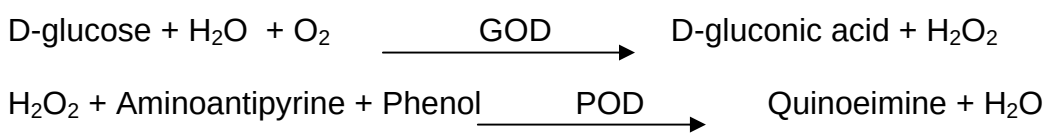
Ammonia was estimated in the neutralized PCA extracts of serum, liver and brain by the method of Ratnakumari and Murthy (1990). To 1ml of the supernatant 1.5ml of phenol-nitroprusside reagent (containing 50g of phenol and 250mg sodium nitroprusside in 3.75 liters of water) and 2ml of sodium hypo-chlorite reagent (8.4g sodium hydroxide, 8.92g Disodium hydrogen orthophosphate and 10ml of 5%sodium hypo-chlorite per litre) were added. After

20 minutes at room temperature, the colour intensity was measured at 630nm against distilled water blank. Ammonium chloride (0.1-1.0 mmoles) was used as standard.

2.6.2 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.



1ml of the working enzyme reagent was added to the 20 μl sample. It was mixed well and incubated at 37^0C 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 6ml with de-ionized water. The tubes were kept in a water bath at 100^0C for 10 minutes, cooled under tap water and then the absorbance was measured against blank (urea reagent + DAM reagent) at 520 nm.

2.6.3 Determination of activities of aminotransferases

2.6.3.1 Aspartate aminotransferases (AST)

Activity of aspartate aminotransferase was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500μl of 160 mM potassium phosphate buffer (pH 7.4), 25 μl of 20mM 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 (0.5mg protein/ ml), and 20 μl of sample. The reaction was started by the addition of α– KG. 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 μmol/g wet weight/h while serum activity was expressed as μmol/ml/h**.**

2.6.3.2 Alanine aminotransferase (AAT)

Activity of alanine aminotransferase was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500μl 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 (0.5mg protein/ ml), and 20 μl of sample. The reaction was started by the addition of α– KG. 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 μmol/g wet weight/h while serum activity was expressed as μmol/ml/h**.**

2.6.4 Determination of protein content

Protein content in serum and liver samples was estimated by Biuret method (Varley, 1969). Bovine serum albumin was used as standard.

2.6.5 Determination of prothrombin time

20μl of heparin was taken in 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. Prothrombin time was determined by using the diagnostic kit, Liquiplastin, supplied by Tulip

Diagnostic (P) Ltd., India. 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^{o}C for 5 min. To this tube, 0.2 ml of Liquiplastin reagent (prewarmed at 37^{o}C) was added, contents were mixed and simultaneously a stop watch was started. The time required for the appearance of the first fibrin strand was recorded.

2.6.6 Estimation of bilirubin:

Bilirubin was estimated by using the commercially available standard kit obtained from Glaxo, India. To 0.2 ml of serum, 1 ml of Diazo A and 0.1 ml of Diazo B were added. For direct bilirubin estimation activator was not added while for estimation of total bilirubin, 1ml of activator was added and the contents were made up to 4.8 ml with water and incubated for 1 minute for direct bilirubin and 5 minutes in dark for total bilirubin. The absorbance was measured at 540nm.

2.6.7 Estimation of albumin:

Albumin was estimated by using the commercially available standard kit obtained from Glaxo, India. To 0.01ml of serum, 1ml of buffered dye reagent and 2ml of distilled water were added and mixed. The absorbance was immediately measured at 630nm.

2.6.8 Estimation of free fatty acids in serum:

Free fatty acids in serum were estimated by the method of Tinnikov et al., (1999). The working solution consisted of 10ml of 0.5mM $Cu(NO_3)_2$, 10 ml of 1 ml triethanolamine(TEA) and 3.5 ml of 1M NaOH diluted with double distilled water to 100 ml and 33g of NaCl was added and the pH was adjusted to 8.1. DCP (diphenylcarbazide) was prepared in ethanol (0.4%) and was stored under liquid nitrogen. Palmitic acid standards were prepared in CHM (Chloroform-heptane (4:3, v/v),2% methanol) with the 0.2 M working standard . A 10 μl volume of plasma was added to 1 ml of CHM solution. A 10 μl volume of double distilled water was added to all the test tubes in standards. The samples were treated in the same manner. All the tubes were vortexed on a multivortexer for 2 minutes. Silicic acid (50mg, activated overnight at 120^0C) was then added , vortexed again for 4 minutes, and centrifuged for 3-5 minutes at 2000Xg.The upper 0.8 ml supernatant was transferred with a glass pipette to another tube containing 0.4 ml of Cu-TEA solution, vortexed for 5 minutes and centrifuged for 3 minutes at 2000g. A 0.6 ml volume of the upper phase from each tube was transferred to a new tube. This upper phase was then evaporated under nitrogen at 37^0C in a fume hood, 0.4 ml of ethanol added, and the tubes were again placed into a water bath for 15 minutes. To ensure complete uptake by the ethanol, tubes were vortexed for 5 minutes. To each tube, 0.2 ml of warm (room temperature) DPC solution was added immediately. The tubes were vortexed for 15 sec and absorbance read at 490nm after allowing the colour to develop for 15 minutes.

2.6.9 Estimation of free tryptophan levels in serum:

Free serum tryptophan levels were estimated by the method of Denkla and Dewey (1967) after ultrafiltration of the serum. 20 µl of serum was mixed with 1.8ml of TCA/$FeCl_2$ and centrifuged at 20,000 rpm for 10 minutes. The supernatant was then mixed with 200 µl of formaldehyde and then heated at 99^0C to 101^0C for 1 h. The tubes were cooled to room temperature and read in a spectrofluorometer with excitation at 373 nm and emission at 452 nm.

2.7 SDS-PAGE and Coomassie staining of proteins:

SDS-PAGE analysis of serum protein of normal and thioacetamide treated rats were carried out on vertical slab gels according to the method of Laemmli (1970). Samples containing 100 µg of protein were dissolved in sample buffer containing 1% SDS (W/V), 5% β-mercaptoethanol (V/V) 0.003% bromophenol blue(W/V) and 12% glycerol(V/V), in 0.063 M Tris-HCl pH 6.8. The samples were boiled at 100^0 C for 5 minutes and subjected to electrophoresis. The stacking gel contained 4.5% polyacrylamide in 0.125 M Tris-HCl pH 6.8 and the resolving gel contained 10% polyacrylamide in 0.375 M Tris-HCl pH 8.8. The ratio of acrylamide to N,N,N',N'- methylene bis acrylamide was 29.2:0.8. Proteins were electrophoresed at constant potential difference (80) volts with an electrode buffer containing 0.025 M Tris-HCl, 0.192 M Glycine and 0.1 % SDS with pH8.5. Molecular weight markers were run simultaneously with the samples. The gel was observed by coomassie staining. Protein was estimated by the method of Bradford (1976).

2.8 Histopathology:

Liver histology was analysed in control and in thioacetamide treated rats at different time periods (6, 12, 18, and 24 h) after the administration of the 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 fixed in Bouin's fluid (Picric acid: Formaldehyde: Glacial acetic acid (15:5:1) for one and half hour at room temp and subsequently rinsed in cold 70% ethanol, dehydrated through graded series of ethanol and finally embedded in paraplast. After embedding in paraplast, the excess paraplast was trimmed off and square shape blocks were made to be fixed on the microtome. 8 µ sections were cut using a rotary microtome. The slides were applied with Meyer's albumin (1:1 egg albumin:

glycerol) and then the histological sections cut were placed on the slide and kept at 40-45^{0}C till the slides became dry. Care was taken so that there were no air bubbles in between the sections.

The sections were deparaffinised in Xylene (3 changes, 5 min each), then hydrated through decreasing concentrations of alcohol (100%, 90%, 70%, 2 changes of 5 minutes in each). Sections were then stained in haematoxylin (3 min). Slides were washed with running tap water for 10 minutes to remove the excess stain. Subsequently sections were counterstained in Eosin (8 min), then dehydrated with increasing concentration of alcohol (70%, 90%, 100%, 2 changes of 5 min in each). The slides were finally passed through xylene (3 changes, 5 min each) and mounted with DPX mountant. Sections were then observed and photographed for histological changes in a compound microscope fitted with Nikon automatic camera. The same procedure was adopted for the brain samples also.

2.9 Amino acid Analysis:

2.9.1 Sample preparation for amino acid analysis.

20% homogenates of the different regions of rat brain were prepared in 10 % ice-cold trichloroacetic acid solution. The homogenates were then centrifuged at 14000rpm for about 30 minutes. The supernatants were then filtered through 0.22 micron filter. 20 µl of this was injected into the HPLC. For Serum 100 µl of serum was taken and 100 µl of 10 % ice cold trichloroacetic acid solution was added and then centrifuged at 14000 rpm for 30 minutes. The supernatant was

filtered through 0.22-micron filter and then further processed as required. The chromatographic conditions required for estimating the amino acids was according to the method of Rosenlund (1990).

Mobile phase for amino acid analysis. Sol A: The buffer A contains 140mM sodium acetate along with 1 ml of trimethylamine (pH 6.4) and 127 ml of acetonitrile and made up to 2 litres of water. Sol B: The buffer B contains 60 % Acetonitrile in water. The above mobile phases are filtered through 0.45-micron filter and degassed well. The column used was C_{18} reverse phase (250 X 4.6 mm), 5μ particle size. Absorbance was recorded using the photodiode array detector at 254nm. A linear gradient program was run for 70 minutes with temperature set at 46^0 C.

2.10 HPLC Analysis of tryptophan metabolites:

2.10.1 Determination of serotonin, tryptophan and 5-hydroxyindoleacetic acid.

The brains were removed and chilled on ice. All the brain regions were homogenized in 0.15% perchloric acid containing 0.025% EDTA (pH 3). The samples were centrifuged at 14,000 rpm for 30 minutes at 4^0 C. The supernatant was filtered through (0.22 microns) filter and were assayed on the same day or stored at -80^0C. This supernatant was used for the estimation of serotonin, tryptophan and 5-hydroxyindole acetic acid.

Serotonin, tryptophan and 5-hydroxyindole acetic acid in CC, CE and PM, brain were estimated by the method of Pawlak et al. (2000). The high - performance liquid chromatography system consisted of LC-10AT pump, a fluorescence detector (RF-10 AXL) and a 5µ C_{18} Luna ODS 2 reverse phase column (250 mm X 4.6 mm i. d.). Excitation and emission wavelengths were set at 285 and 345nm. Tryptophan was eluted with 10mM sodium acetate (pH 4) and 10% methanol and the gain on the detector was set to 4. Corresponding standards were run for quantification purposes. The flow rate was set at 2.0 ml per minute. The quantification was done by peak area analysis.

2.10.2 Determination of Kynurenine:

HPLC was carried out using C_{18} 3.5µ Kromasil reverse phase (150X 3.5 i.d.) column and a mobile phase prepared by mixing 5ml of acetonitrile with 235 ml of 0.1M acetic acid and 0.1M-ammonium acetate (pH 4.65) at a flow rate of 0.5 ml per minute. The absorbance of the column effluent was monitored at 365nm. The quantification was done by peak area analysis using Shimadzu Chromatopac C6RA. Samples were homogenized in 2.4 M perchloric acid and centrifuged at 14000 rpm for 20 minutes. Twenty microlitres of this clear filtered supernatant was injected into the Rheodyne injector. Kynurenine standard was used for quantification purposes. The same procedure was also followed for analyzing the serum kynurenine levels. The quantification was done by peak area analysis.

2.10.3 Indoleamine 2,3-dioxygenase assay:

Tissue homogenates of different regions of brain viz., CC, CE and PM were prepared in ice cold 0.14M KCl/20mM potassium phosphate buffer (pH 7) with a Teflon homogenizer. The homogenates were centrifuged at 25,000g for 30 minutes at 4^0C. The supernatant of this was used for the enzyme assay of the indole amine 2,3 dioxygenase (IDO) activity. The IDO enzyme activity was quantified by the conversion of L-tryptophan to L-kynurenine by the modified method of Saito et al. (1991). The standard mixture consists of 0.5 mg protein, 50 mM potassium phosphate buffer (pH 6.5), 25 µM methylene blue, 20 mM ascorbate, 300 µg of catalase and 7.8mM tryptophan in a final volume of 1 ml. During the initial standardization, concentrations of the various components of the reaction mixture were altered until an optimal concentration was found. The reaction mixture was incubated for 60 minutes in a water bath with shaking (100 stokes/minute) and then terminated by the addition of 0.5 ml of 10% TCA. Incubation was continued for another 45 minutes at 55^0C to hydrolyze N-formyl kynurenine to kynurenine. The samples were centrifuged at 12000 g for 10-15 minutes and the product formed was estimated by HPLC. For the controls, zero time samples were used. The activity was expressed in nmol/g wet weight/hr. Protein was estimated by the method of Bradford (1976).

2.10.4 Determination of 3-Hydroxykynurenine:

20 % homogenates of different regions of brain were prepared in 2.4 M perchloric acid and centrifuged at 14000 rpm for 20 minutes. HPLC was carried

out using C_{18} 3.5µ Kromasil reverse phase (150mmX 3.5mm i.d.) column and a mobile phase prepared by mixing with 5% methanol and 95% water containing 5mM ammonium acetate. The flow rate was set at 0.75ml/min and the absorbance was read at 340nm. The chromatographic conditions used here were according to the method of Okuno and Kido (1991).

2.10.5 Determination of Kynurenic acid levels:

Frozen brain tissues were thawed and homogenized (1:10 wt/vol) in distilled water. 200µl of the tissue homogenate was acidified with 50µl of 6% perchloric acid and centrifuged at 13000 g for 10 minutes. Then 125 µl of the resulting supernatant was diluted with mobile phase (1:1 wt/vol) and 100 µl was injected to a 125X 4.6 mm C_{18} reverse phase HPLC column. Kynurenic acid was eluted at 1ml/minute with a mobile phase containing 0.2 M zinc acetate and 3.5 % acetonitrile, titrated to pH 6.2 with glacial acetic acid. Kynurenic acid was detected fluorometrically (excitation wavelength 344nm and emission wavelength 398**)** using a shimadzu spectrofluorometer. The chromatographic conditions used above were according to the method of Ceresoli-Borroni and Schwarcz (2000). The flow rate was set at 1ml/minute.

2.10.6 Determination of Anthranilic acid:

Frozen brain tissues were thawed and homogenized in perchloric acid 0.4M (1:10 w/v).The samples were sonicated and centrifuged for 20 minutes at 3000g. 50µl of the resulting supernatant were subjected to HPLC analysis. The

eluant was prepared by mixing an aqueous solution of 20mM of sodium acetate/ acetic acid (pH 5.5) with methanol in a 90:10 (v/v) proportion. The eluant was filtered through 0.22 micron cellulose acetate filter and degassed before use. The flow rate was 1ml/minute. The separation was obtained using the column Lichrospher C_{18} reverse phase HPLC column (125X 4.6 mm) and fluorescence detector with excitation at 316nm and emission at 420nm.

2.11 Immunohistochemistry studies:

Immunohistochemistry (ICC) for Quinolinic acid was carried out as per the following procedure. The sections were deparaffinised and rehydrated in a gradient of decreasing concentrations of alcohol and finally in PBS containing 1% tween (pH 7.6). The sections were then treated with normal goat serum (1:10 dilution). Primary antibodies against quinolinic acid was applied to the sections at 1:80 dilution and incubated at 4°C for 48 h in a humid chamber. The sections were then washed with PBS thrice at 5 min interval and incubated at room temperature for 1 h with HRP conjugated anti-mouse IgG. The sections were then washed with phosphate buffer (PB) and incubated with 0.05% 3,3'diamino benzidine tetra hydrochloride with 0.01% H_2O_2 in 0.05M Tris-HCl (pH 7.6) for 10 min in a dark chamber at room temperature. The slides were then rinsed with PB, dehydrated through graded alcohol series, cleared in xylene and mounted in DPX. All the chemicals and secondary antibodies used for ICC were part of the kit components supplied by Bangalore GENEI Pvt. Ltd. (Bangalore, India)

2.12 Electron microscopic studies:

Preparation of tissue for Transmission Electron Microscopy

Tissues from different regions of brain after perfusion were cut into 400μm thick sections with a vibratome. Slices were washed in cold 0.1M sodium cocodylate buffer and kept in 2.5% glutaraldehyde in 0.1M cocodylate buffer until further processing. When processing was resumed, slices were washed in cold cocodylate buffer and were then post fixed in 1% osmium tetraoxide in cold cocodylate buffer for 1hour. After osmium tetroxide step, 400μm thick sections were washed in 0.1% cocodylate buffer. 2x2mm 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 (0.5μm thick) sections were cut out with an ultra microtome, stained with toluidine blue and examined by light microscope for an over view. Ultra thin 70-90nm thick sections were then cut, picked up on 200 mesh copper grids, double stained with uranyl acetate and lead citrate and observed under (Model: Hitachi, H-7500) electron microscope.

2.13 Blood-brain barrier studies:

2.13.1 Histological evaluation:

The structural integrity of the blood brain barrier was studied in normal and TAA treated rats. A 2% solution of Evans blue was given at a dose of 3 ml/ kg (60 mg/ kg) i.p to both normal and TAA treated rats and transcardinal perfusion was performed with saline before decapitation. In TAA treated rats, it was

administered after 12h after the second dosage of TAA administration. Both the animals were perfused with saline before decapitation. The regions were separated, fixed, embedded and then cut into 6 micron sections before observing under the microscope. The blood brain barrier is a regulatory interface that allows the entry of only specific substances into the brain. An intact blood brain barrier normally prevents the entry of Evans blue from staining the brain. Any staining of the brain, therefore, would be reflected as either an increase in the permeability or damage to the blood brain barrier.

2.13.2 Spectrophotometric method:

The integrity of BBB was performed by quantitative measurement for EB content (Mikawa, 1996*)*. Before decapitation, rats were perfused with saline to remove intravascular EB dye. The brain was quickly removed and dissected into three regions; cortex, cerebellum and pons medulla. The samples were examined for Evans blue albumin extravasation and the extent and intensity of the staining. Each brain region was weighed and the samples were then homogenized with 2.5 ml phosphate-buffered saline (PBS) and mixed with 2.5 mL 60% trichloroacetic acid to precipitate protein. The samples were centrifuged for 30 min. at 1000*g* and the supernatants were measured at 610 nm for absorbance of EB by using a spectrophotometer. EB is expressed as micrograms per milligram of brain tissue against a standard curve.

2.14 Estimation of Antioxidant status and Oxidative stress:

2.14.1 Lipid peroxidation

The extent of lipid peroxidation was estimated in brain homogenates by measuring the levels of malondialdehyde (MDA) formation using the thiobarbituric acid modified method of Okhawa et al.,(*1979).* Briefly, 10% (w/v) homogenates of the above mentioned brain regions, were prepared in 1.15% KCl by using a Teflon homogenizer. 300µl of this homogenate was added to 700 µl of water and the mixture was incubated for 30 minutes at 37^0C. Two ml of thiobarbituric acid (0.375% TBA in 15% TCA) was added and the mixture was kept at 95^0C for 30 minutes. To avoid evaporation of the samples, tubes were closed with marbles. After cooling at room temperature, the volume was checked and readjusted. The samples were centrifuged at 5000 rpm for 10 minutes and the absorbance of the supernatant was measured at 532nm. MDA values were calculated by using the standard 1,1,3,3, tetramethoxy propane obtained from Sigma-Aldrich as standard.

2.14.2 ROS levels:

The levels of ROS were determined by the method of Montoliu et al., (1994). An appropriate volume of freshly prepared tissue homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 µM Dichlorofluoroscein diacetate for 60 minutes at 37 ^{0}C. The dye loaded samples were centrifuged at 12500g for 10 minutes at 4^0C. The fluorescence measurements were performed with a Hitachi spectrofluorometer at 488nm for excitation and 525nm for emission wavelengths.

2.14.3 Determination of total nitric oxide levels by estimating nitrites and nitrates:

Nitrites and Nitrates in the homogenates of different regions of brain viz., Cerebral Cortex (CC), Cerebellum (CE) and Pons Medulla (PM) were estimated by the method of Sastry et al., (2002). This method involves a modification of the acidic Griess reaction for the colour development after the reduction of nitrate with copper-cadmium alloy (the alloy was a kind gift from Sastry, K.V.H, IVRI, Izzatnagar, UP India). Before use, the copper-cadmium filings were washed in the 50 mM carbonate buffer (pH 9.0) and dried on the filter paper. The original Griess reaction is useful to measure only nitrite levels. However, under physiological conditions, nitric oxide is converted initially to nitrite then to nitrate by nitrite reductase. In the modified method, the copper-cadmium alloy converts the nitrate to nitrite. The difference in the nitrite levels before and after conversion would then indicate the amount of nitrate.

In this procedure, 10% (w/v) tissue homogenates were prepared in 1.15% KCl. Tissue homogenates (100µl) and serum (100µl) were taken into separate tubes and 400 µl of 50mM carbonate buffer (pH 9) was added, followed by the addition of approximately 150 mg of washed copper-cadmium filings. The tubes were incubated at room temperature for 1hour with shaking. The reaction was stopped by the addition of 100µl of 0.35M sodium hydroxide and 400µl of 120mM zinc sulfate and vortexed. After 10 minutes, the tubes were centrifuged at 4000g for 10 minutes. 150µl of this clear supernatant was taken and the components of

the Griess reaction mixture (75µl of 1%sulfanilamide prepared in 3N HCl and 75µl of 1%N-Napthylethylene diamine prepared in water) were added with gentle mixing each time. The tubes were again incubated for 10 minutes and the absorbance was determined at 545nm. For assaying the nitrite levels, the same steps were followed except for the addition of copper-cadmium alloy.

2.14.4 Immunohistochemical detection of 4-hydroxynonenal-modified proteins:

Different regions of brain fixed in 4% buffered formaldehyde in PBS for 24h were dehydrated, embedded in paraffin and sectioned. These paraffinized sections were deparaffinised, rehydrated, and in a gradient of decreasing concentrations of alcohol and finally in PBS containing 1% tween (pH 7.6). Sections were then treated with 3% hydrogen peroxide in methanol (v/v) for 20 min. in order to block endogenous peroxidases. Then the sections were incubated with a rabbit polyclonal antibody (anti HNE at a dilution of 1:500) (Alpha diagnostic International, San Antonio, TX, USA) in PBS (pH 7.4) containing 0.1% Tween -20 and 1% bovine serum albumin in a moist chamber at 4^0C overnight. The sections were thoroughly washed with PBS (10mM sodium phosphate, 0.15 M NaCl, pH 7.4) containing 0.5% Triton X- 100 (v/v) and treated with Horse radish peroxidase –linked anti rabbit IgG secondary antibody for 60 min. at room temperature and then washed with PBS and developed using diaminobenzidine (Bangalore Genei, Bangalore, India), mounted with DPX and examined under Nikon photomicroscope.

2.14.5 Antioxidant enzyme activities:

2.14.5.1 Preparation of tissue extracts for the measurement of enzyme activities of glutathione peroxidase, glutathione reductase and catalase.

Different regions of the brain were homogenized in cold 50mM phosphate buffer (pH 7) containing 1mM EDTA to give 10% homogenate. The homogenates were centrifuged at 10,000 rpm for 10 min. at 4^0 C. The supernatants were separated and used for the enzyme assays. Protein was estimated by the method of Bradford (1976).

2.14.5.2 Glutathione peroxidase activity (GPx):

Glutathione peroxidase (GP_X) (EC 1.11.1.9) activity was determined by the method of Paglia and Valentine, (1967). Each 3 ml assay volume contained 0.2 ml of sample, 0.05 M phosphate buffer (pH 7.0) containing 5 mM EDTA, 0.14 mM NADPH , 3.0 mM sodium azide , 1mM GSH , 1 U glutathione reductase and 100 µl H_2O_2 . Changes in absorbance were recorded at 340 nm for 5 minutes on Hitachi spectrophotometer and the enzyme activity was calculated as nmoles of NADPH oxidized/ minute/mg protein .

2.14.5.3 Glutathione reductase activity (Gr):

The activity of Glutathione reductase (EC 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1975). Briefly the assay mixture contained 200 mM phosphate buffer pH 7.0, 2mM EDTA, 2 mM NADPH and 20 mM GSSG and a known amount of enzyme source. Disappearance of NADPH was measured immediately at 340 nm against blank containing all the components except the enzyme at 10 s interval for 3 minutes. The enzyme activity was expressed as nmoles of NADPH oxidized/ minute/mg protein.

2.14.5.4 Catalase activity:

Catalase (EC1.11.1.6) was assayed by the method of Claiborne (1985). Briefly the assay mixture contained 2.4 ml of phosphate buffer (50 mM pH 7.0), 10 µl of 19mM H_2O_2 and 50 µl of enzyme source. The decrease in absorbance was measured immediately at 240 nm against blank containing all the components except the enzyme at 10 second intervals for 3 minutes on a Hitachi spectrophotometer. The enzyme activity was expressed in µ moles of H_2O_2 consumed/minute/per mg protein.

2.14.5.5 Determination of Superoxide dismutase activity:

For measurement of Superoxide dismutase enzyme activity, different regions of brain homogenates viz., CC, CE & PM were centrifuged at 2300g for 15 minutes and then the supernatant was pre-incubated at 25^0 C for 10 minutes in Tris buffer to make up a total volume of 980µl before the addition of 20 µl of 10

mM pyrogallol solution in 10 mM HCl. The total SOD activity was measured spectrophotometrically at **420**nm. A unit of enzyme activity (U) is defined as the amount of enzyme necessary to inhibit the superoxide oxidation of 0.2mM pyrogallol. The protocol followed was according to the method of Bay et al. (1999).

2.14.6 Glutathione estimation:

The determination of GSH (reduced form of glutathione) and GSSG (oxidized form of glutathione) was performed by the method of Hissin and Hilf (1976). To 0.5 ml of the tissue extract (10,000g supernatant), 4.5 ml of the phosphate-EDTA buffer, pH-8, was added. The final assay mixture (2.0ml) contained 100µl of the diluted tissue supernatant ,1.8 ml of phosphate-EDTA buffer and 100µl of the orthopthaldehyde (OPT) solution containing 100µg of OPT. For GSSG estimation a 0.5 ml portion of the tissue extract was incubated at room temp with 200 µl of 0.04 NEM for 30 minutes to interact with GSH present in the tissue. To this mixture, 4.3 ml of 0.1 N NaOH was added. A 100 µl portion of this mixture was taken and to it 1.8 ml of 0.01 N NaOH and 100 µl of the orthopthaldehyde (OPT) solution containing 100 µg of OPT were added. After thorough mixing and incubation at room temperature for 15 minutes, the solution was transferred to a quartz cuvette. Fluorescence at 420nm was determined with excitation at 350nm. The results are expressed as ratio (GSH/GSSG) of reduced (GSH) and oxidized (GSSG) forms of glutathione.

3. ANIMAL MODEL

3.1 Development of an animal model

Fulminant hepatic failure (FHF) is a condition with a sudden onset of necrosis of hepatocytes and degeneration of liver without any prior established liver disease. When liver function deteriorates or blood is shunted past the liver, hyperammonemia results and there is an associated deterioration in the brain function, a disorder known as Hepatic encephalopathy. These neurological changes develop within hours and frequently results in death from increased intracranial pressure due to brain edema.

An understanding of the mechanisms involved in the pathogenesis of HE can be obtained by investigations with samples obtained from HE patients. This approach however, has significant limitations such as a) ethical restriction; b) difficulty in finding the material and even if the material is available, postmortem changes would be observed due to the lapse of time between the collection of the sample and performance of the experiment. Further, most human patients may have additional complications which could interfere with important observations & other evaluations; c) drugs administered during the course of treatment may interfere with the biochemical parameters in the sample and collectively make it difficult to infer whether the results obtained are really due to the pathological condition or due to the drugs administered; d) analysis at uniform intervals of time is not practicable if the study is based on the human samples and e) compounds with potential therapeutic value can not be used without prior assessment of their effect. These limitations necessitate

development of an animal model which closely mimics human condition so as to facilitate the study of alterations in the cerebral functions of different regions of brain and the mechanisms involved for these changes may be more revealing and rewarding for better understanding than in human samples. In addition, animal models have an advantage of being easily bred and handled in normal laboratory conditions and also availability of enough samples for the biochemical and morphological characterization. Further, different experimental parameters can be precisely regulated and altered so as to allow unambiguous interpretation of results obtained.

3.1.1 Features of an ideal animal model

Terblanche and Hickman (1991) have proposed the following criteria 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 liver dysfunction.

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. Large animal model: Most artificial liver support systems require large animal models such that blood and tissue analysis can take place serially

Chapter 1

Animal Model

as treatment is being assessed. This is more relevant to humans and makes the scale up for use in man, less problematic.

6. Therapeutic window: Time should be available between insult and death such that the treatment can be investigated and assessed for its effect.

7. Minimal hazard to handling personnel.

3.1.3 Types of Animal models available for FHF:

Two different approaches have been used to study the animal models for FHF.

1. Surgical procedures

2. Administration of specific hepatotoxins

3.1.3.1 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
Partial devascularization	Pig	Late hypoglycemia, increase in prothrombin time, Minimal increase in AST	De Groot et al., 1987
Total devascularization	Pig	Lethargy and coma, increase in AST and encephalopathy	Hanid et al., 1979

The disadvantage of these models is that they are often non-reversible and do not mimic the clinical patterns seen in man.

3.1.3.2 Animal models based on specific hepatotoxins

Model	Animal	Results	Reference
D- Galactosamine	Rats Rabbit Dogs	Encephalopathy, Increased AST, ammonia, hepatic necrosis, edema.	Kepler *et al*., 1968 Blitzer *et al*., 1978 Diaz Buxo *et al* ., 1997 Chojkier and Fierer,1985 Dixit and Chang., 1990
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 Blei et al., 1992 Reddy et al., 2004
Concanavalin A	Mice and Rats	Increased AST, metabolic acidosis, Centrilobular necrosis	Tiegs, 1997

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

3.1.3.2.1 Thioacetamide-Toxicity

Thioacetamide is a well known hepatotoxin for inducing 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 (Chieli 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 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.

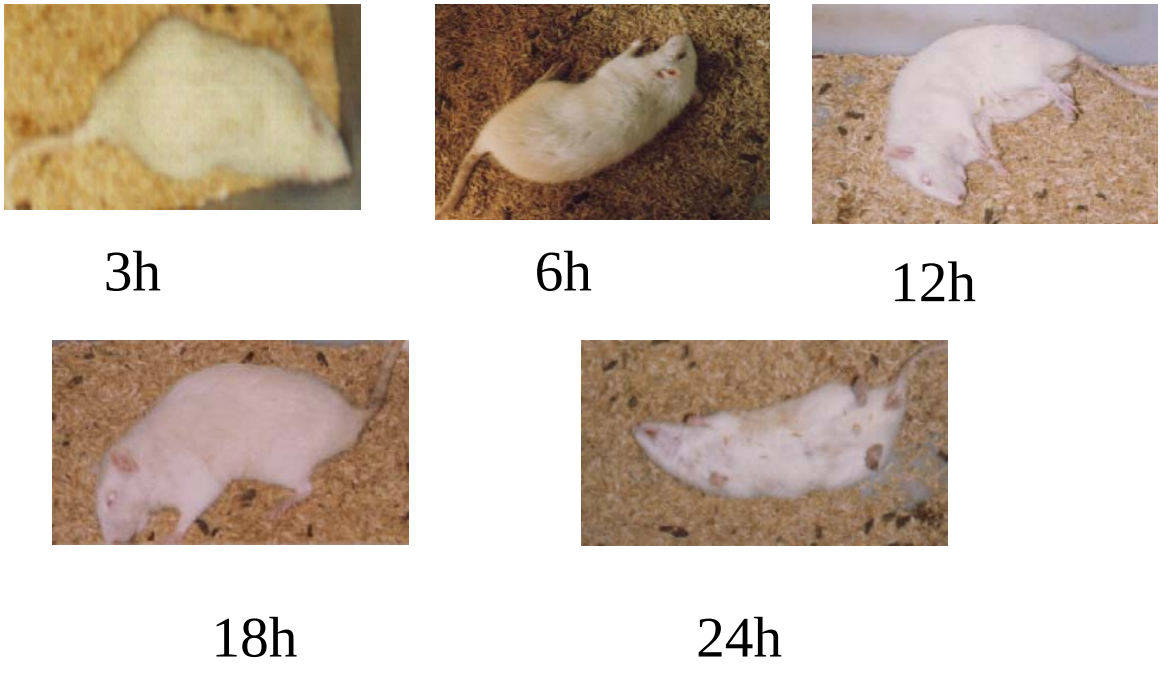


Fig: 3.1. Behavioral changes observed in rats after TAA administration at different intervals of time

A series of neurological and behavioral changes were observed following the administration of second dose of TAA to rats (Fig 3.1). The feeding of the animal decreased considerably after the first dose of TAA and is stopped completely during 12-24h after its second dose. Moreover the animal became progressively inactive and sleepy from 6h after the administration of the second dose. The physical activity of the animal also declined from this point onwards. From 12h after the second dose, the animals showed wobbly gait and loss of rightening reflexes. This change in the gait and loss of reflex was enhanced 18h after the second dose of thioacetamide. Twenty four hours after the drug administration, the animal rests on its back with legs spread out into the air.

Liver function was assessed by monitoring various biochemical parameters such as ammonia, glucose, protein, urea and the activities of aspartate and alanine aminotransferases in the serum and liver. Concurrent studies were also carried out in the brain samples to assess appearance of HE

3.2 Characterization of the developed model:

3.2.1 Liver function tests

3.2.1.1 Glucose

Liver is a vital organ that plays a key role in the homeostasis of blood composition. The regulation of glucose metabolism occurs in the liver which is the chief site for gluconeogenesis, glycogenesis and glycogenolysis. So any damage to the liver will be reflected directly in the blood glucose levels. Hence, to assess the extent of liver damage, glucose levels were estimated in serum as

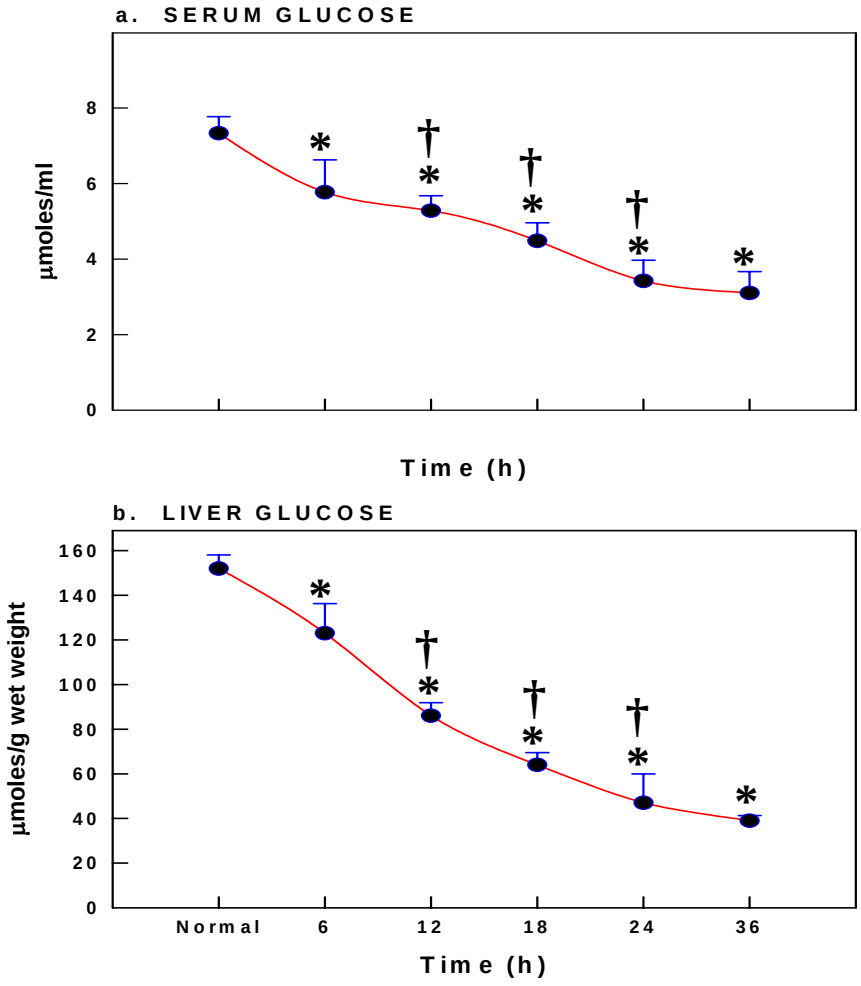
well as in the liver of normal and drug administered rats at different time points. In the present study, serum glucose levels were determined instead of whole blood glucose levels. This was done keeping in mind the fact that blood has different types of cells which also trap glucose. This glucose is 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 3.2a) and liver (Fig3.2b) after thioacetamide administration. In liver the glucose level was decreased by 50 % of the control value within 12h after administration of thioacetamide and by 36h, the glucose level in liver was decreased by almost 75% of the control value.

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 12h, the decrease in liver glucose level (43%) was much higher than those in the serum (28%). At the end of 36h, serum glucose levels decreased to half (58%) while that of liver to one fourth (75%) of the respective control values. The decrease in serum and liver glucose levels could be due to: a) decreased feeding of the animal and b) liver damage caused by thioacetamide. As the animal stops feeding, blood glucose levels would decrease rapidly. During 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 efficient manner due to the significant hepatic dysfunction and necrosis occurring due to thioacetamide treatment (Wagle *et al*., 1976, Hoyumpa and Schenker, 1985).

Fig: 3.2. Levels of glucose in the serum and liver of normal and thioacetamide treated rats at various time points



* Significant over control † Significant over previous time point

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

3.2.1.2 Ammonia

Changes in blood ammonia levels are one of the hall marks of the liver disease. 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 above (3.2.1.1).

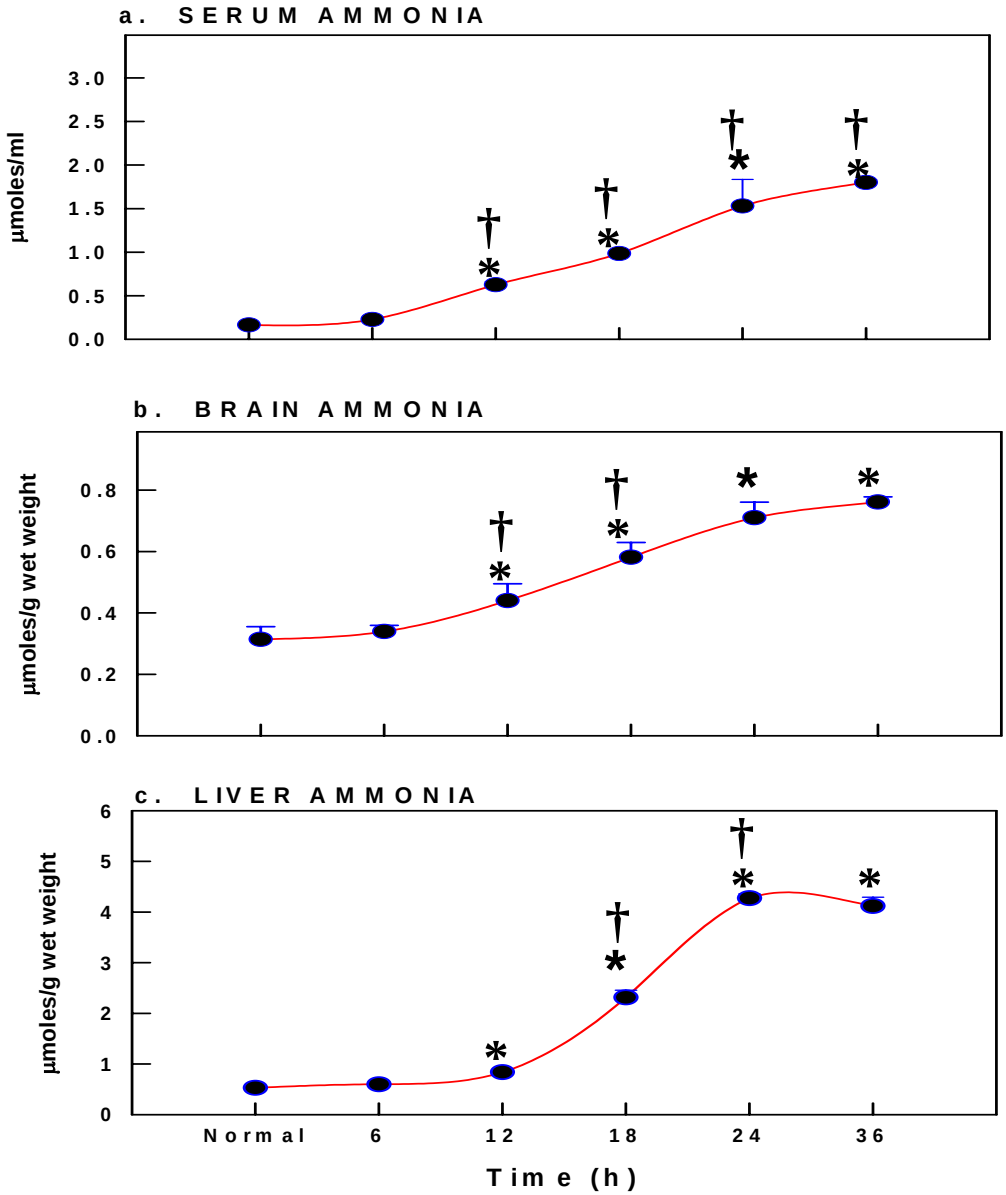
The ammonia levels in serum (Fig 3.3a), brain (Fig 3.3b) and liver (Fig 3.3c) were increased. Ammonia levels in the serum increased 4 fold by 12h after the administration of thioacetamide (second dose) and this increase was progressive. By the end of 36h the serum ammonia levels were elevated by more than 10 fold when compared to controls. Similar results indicating an increase in the blood ammonia levels were reported by Bruck et al., (Bruck et al*.,* 1999 & 2002, Reddy et al., 2004).

The increased ammonia levels observed in the serum (Fig 3.3a) after administration of TAA might be due to 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, majority of which occur in intestine. However, it must be emphasized that this may not be the major source for ammonia in the drug treated animals as these animals stop feeding. Hence, the ammonia should have been produced from some 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. Also, failure of the mechanisms to remove ammonia in the body would also contribute to increase in blood ammonia levels. Increase in the levels of ammonia in the liver of TAA treated animals (Fig 3.3c) suggests such a possibility. This increase in liver ammonia level might be due to decreased detoxification of ammonia to urea in this tissue consequent to metabolic derangements leading to necrosis of liver cells. Hence, in TAA-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 reflected in elevated serum ammonia levels during liver dysfunction (Bruck *et al.,* 1999; 2002). In the present study also, the changes in the liver ammonia levels are reflected in the serum. This is supported by the correlation coefficient of 0.84 between liver and serum ammonia levels (Fig 3.4 b). Once ammonia enters systemic circulation, it will flood the tissues, including brain. Elevation in the brain ammonia levels is well known to be a hallmark of this condition (Albrecht et al., 1990; Norenberg 1998). Results of the present study also indicate a progressive increase in brain ammonia levels in thioacetamide induced fulminant hepatic failure conditions (Fig 3.3b). Moreover, a close correlation (r^2 = 0.97) was seen between serum and brain ammonia levels (Fig 3.4a**)**. Increased levels of ammonia in brain has been considered as the chief culprit in the neurological dysfunction observed in

fulminant hepatic failure conditions (Albrecht 1998, Butterworth et al., 1987, Norenberg 1996;1998).

Fig: 3.3 Levels of ammonia in the a) serum, b) brain and c) liver of normal and thioacetamide treated rats at different time periods

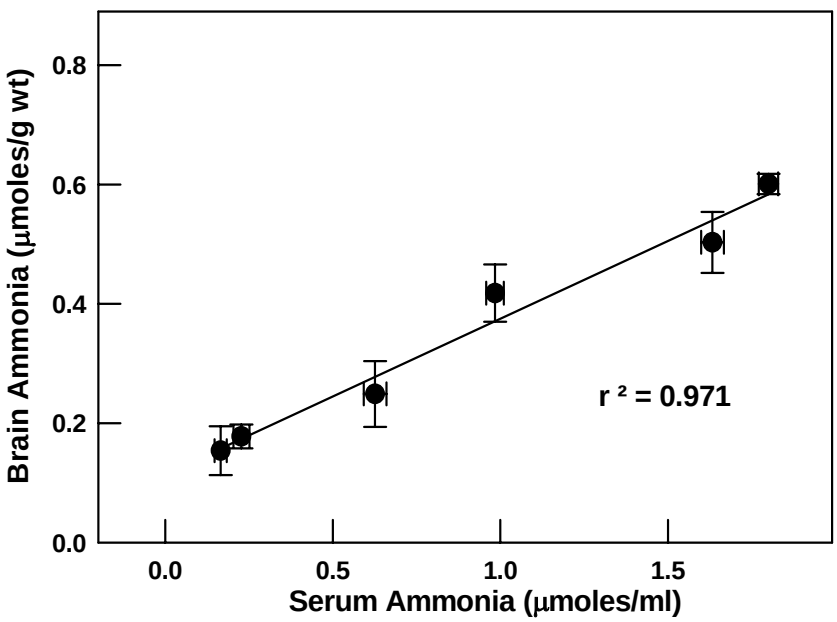


* Significant over control † Significant over previous time point

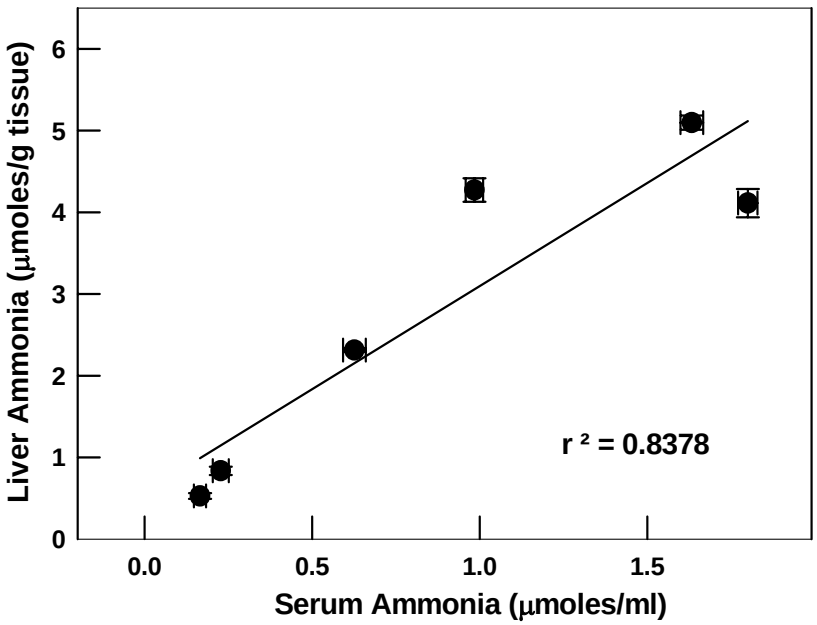
Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Fig 3.4. **Correlation between (a) serum-brain (b) serum-liver ammonia levels in rats with TAA-induced FHF**

a



b

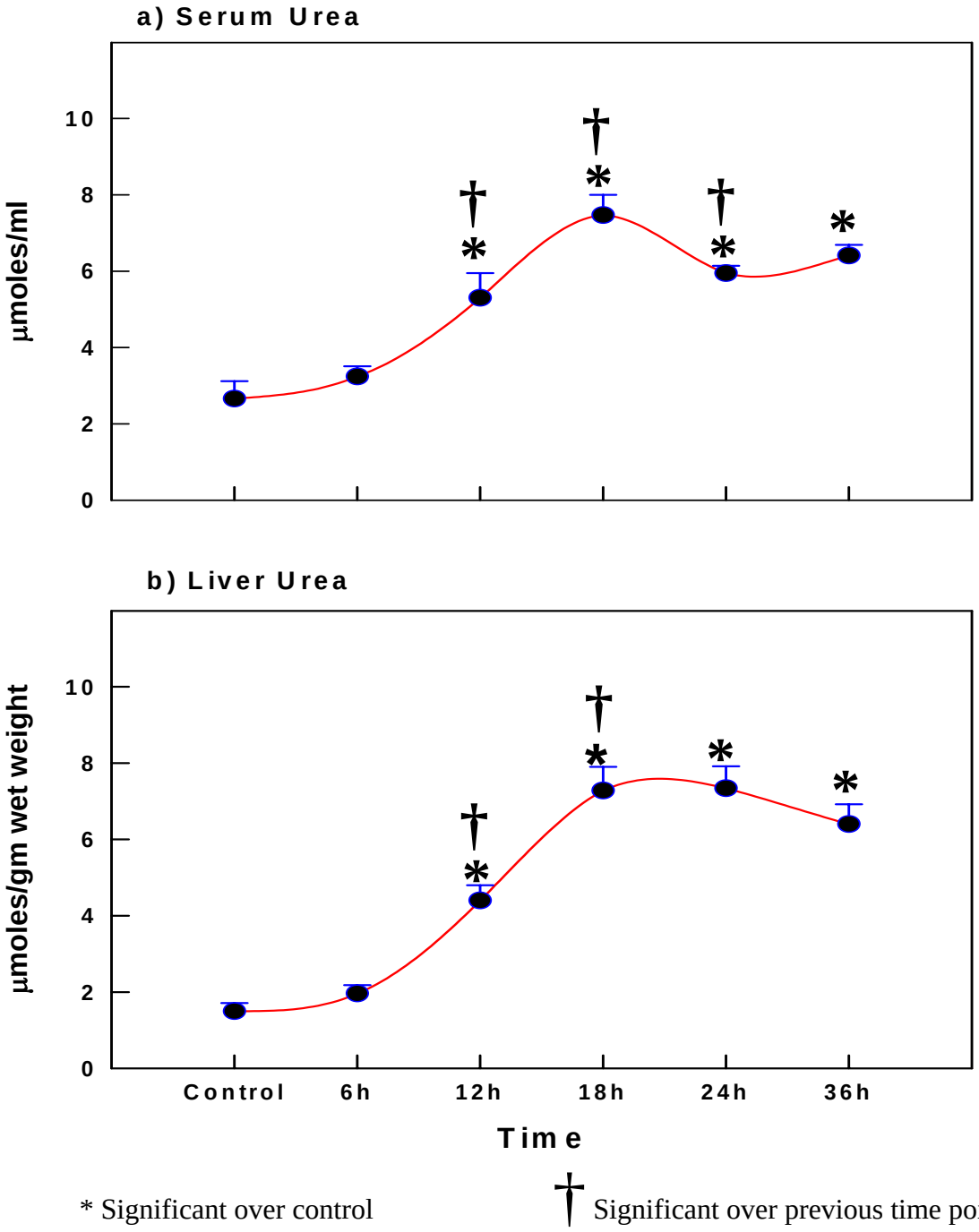


3.2.1.3 Urea

To verify whether the 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. Liver (Fig 3.5b) and serum (Fig 3.5a) urea levels increased significantly in rats administered with thioacetamide. In serum, except for the change at 6h, all other changes at different time periods were significant when compared to the untreated control. There was a 2 fold increase of urea level at 12h time period after thioacetamide injection and was increased by 3 fold at 18h. In liver, the urea level was elevated by 5 fold at 18h after thioacetamide injection and remained same even after 24h.

In summary, urea levels increased both in serum and in liver progressively up to 18h after the administration of thioacetamide with slight decrease thereafter. In liver, there is differential utilization of ammonia in periportal and perivenous hepatocytes. In periportal cells ammonia is converted to urea as these cells are rich in urea cycle enzymes. Perivenous cells convert ammonia to glutamine and these cells are poor in urea cycle enzymes but are endowed with glutamine synthetase. Elevated urea levels in fulminant hepatic failure rats suggested that the periportal cells are spared, while the perivenous cells are affected by thioacetamide toxicity. The spared periportal cells might be responsible for the elevation of urea levels which have reached the maximum by 18h. This might reflect the maximal capacity of the surviving periportal cells to synthesize urea and explains lack of increase in the urea levels thereafter (Haussinger *et al.,* 1984).

Fig:3. 5 **Levels of Urea in the a) serum and b) liver of normal and thioacetamide treated rats at different time periods**



Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

3.2.1.4 Proteins

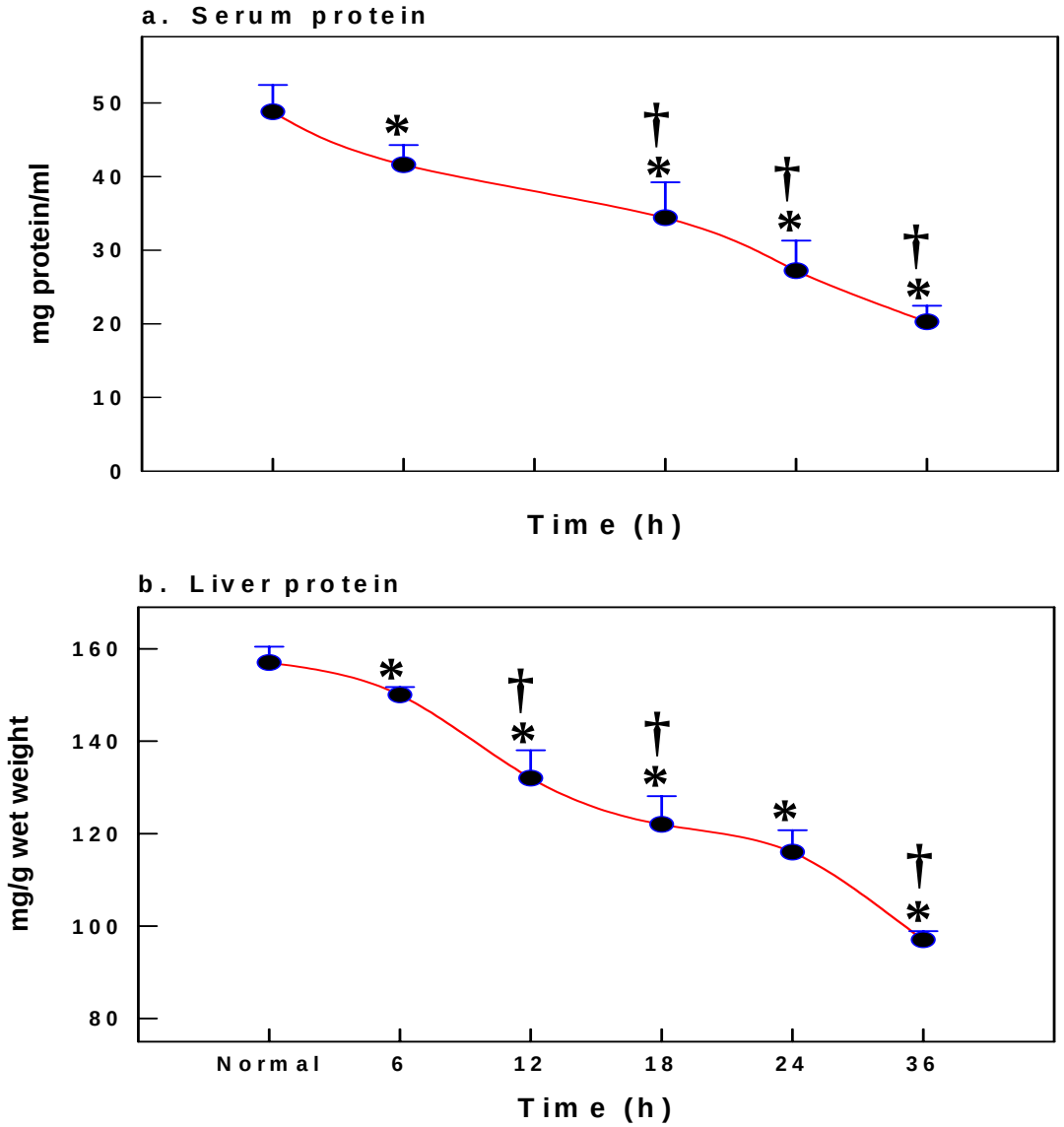
As liver is the major contributor to serum proteins, protein levels were also measured in the liver and in the serum of the animals treated with thioacetamide. The decrease in the liver protein (Fig 3.6b) content by 6h was marginal but statistically significant. At 18h and 24h the levels of protein were reduced by almost 25% of the control value. By 36h a major change of about 40% reduction was observed.

The decrease in the serum protein content (Fig 3.6a) was significant at all the time intervals studied. At 18h there was 30% decrease, 44% at 24h and by the end of 36h 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 because of thioacetamide induced necrosis. Necrosis and 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. These results suggest the possible impaired protein synthesis in the liver of thioacetamide treated rats, leading to decreased serum protein levels. Albumin constitutes about 70-80% of total proteins in the plasma and therefore a decrease in protein level indicates a decrease in albumin levels. Decrease in albumin levels could lead to changes in the oncotic pressure leading to edema. As the proteins contribute substantially to the osmotic pressure of serum, loss of proteins (albumin) would decrease the

osmolality of the serum. This might lead to increased load on the kidneys and also edema of other tissues (Reeba, 1995). The present study showed marked decrease in albumin levels after TAA administration.

Fig: 3. 6 Levels of Protein in the a) serum and b) liver of normal and thioacetamide treated rats at different time periods



* Significant over control † Significant over previous time point

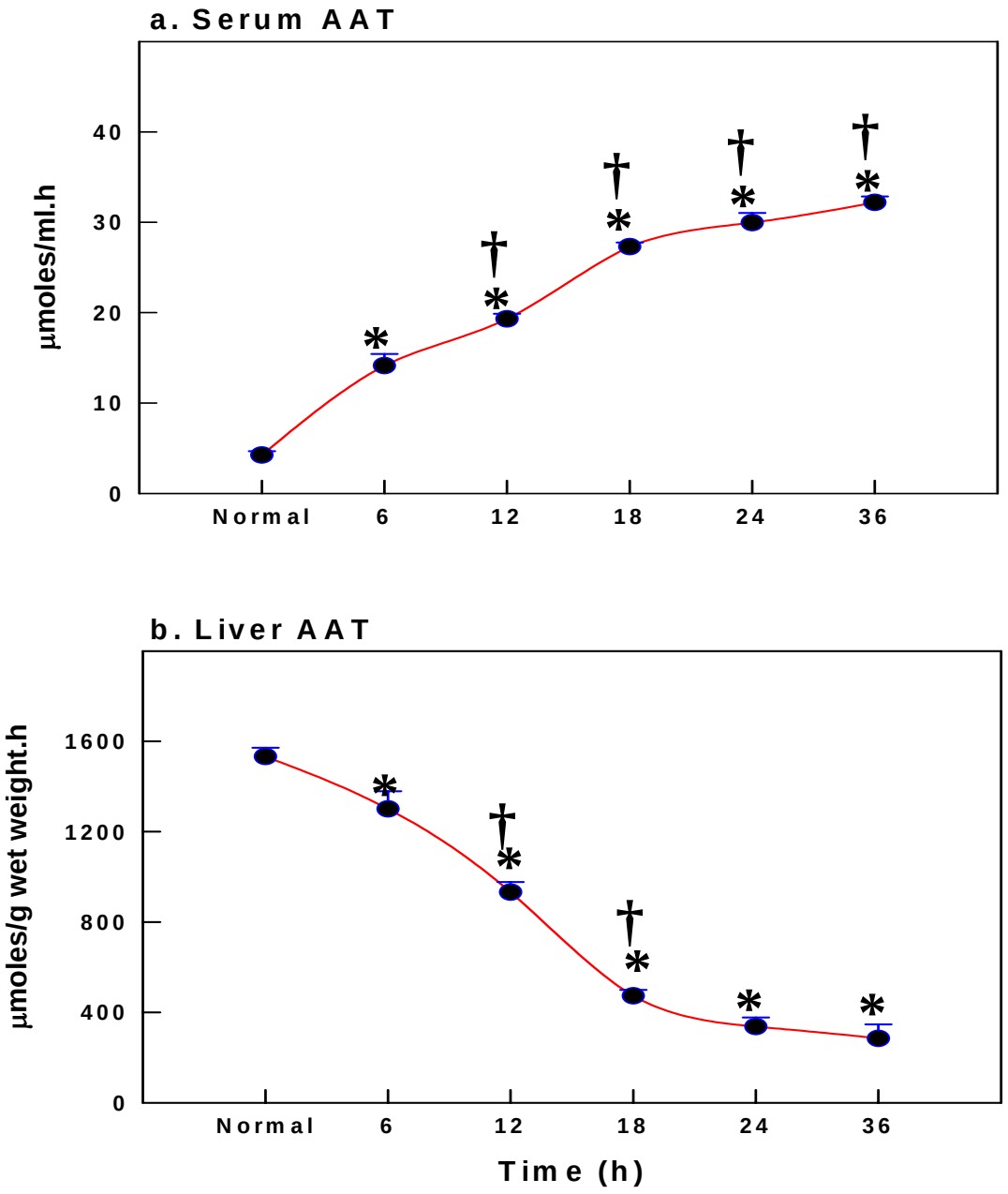
Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

3.2.1.5 Aminotransferases

Alanine aminotransferase (AAT) and Aspartate aminotransferase (AST) activities were estimated in the liver and serum as these two enzymes are considered to be the diagnostic markers for hepatic damage.

A seven fold (661%) increase in serum alanine aminotransferase (AAT) was seen at 36h after the administration of thioacetamide (Fig 3.7a) while an eightfold decrease (81%) was seen in the activity of this enzyme in the liver tissue under these conditions (Fig 3.7b). Similarly, a three fold increase (218%) in the activity of serum aspartate aminotransferase (AST) was observed at 36h after the administration of second dose of thioacetamide (Fig 3.8a) while a 10 fold decrease (90%) was seen in the activity of this enzyme in the liver (Fig 3.8b). Severe liver injury manifested by the elevation of serum AST and AAT were also reported earlier using thioacetamide. (Bruck et al., 1999; 2002). Similar impairment in the liver function of thioacetamide treated rats was reported by Norton and group (Norton *et al.,* 1997). A correlation coefficient (r^2) value of 0.967 and 0.935 was obtained between the changes in liver and serum activities of AAT and AST enzymes (Figs 3.9a&b.). 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.

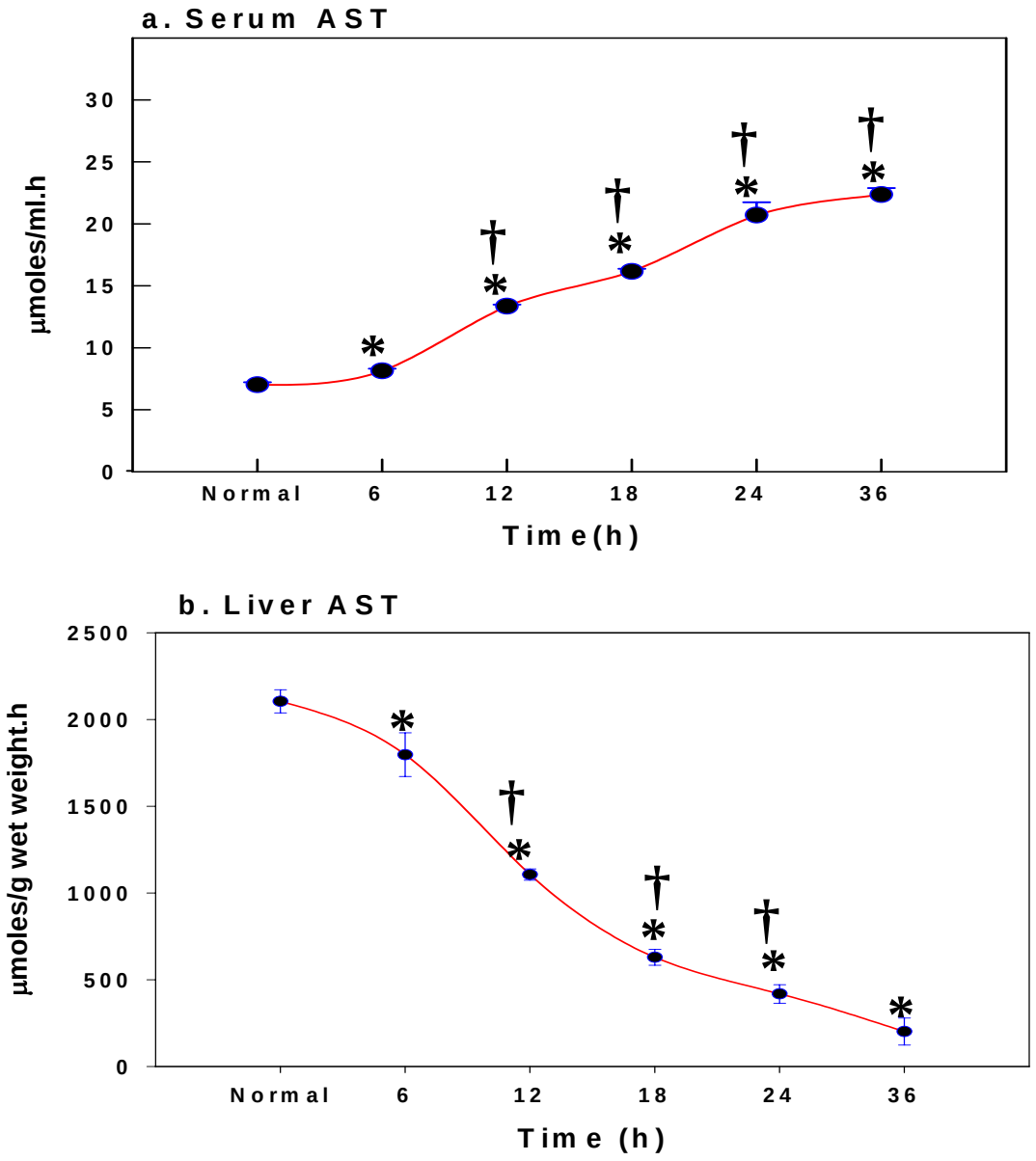
Fig:3.7 Activities of alanine amino transferase in a) serum and b) liver of normal and thioacetamide treated rats at different time points



* Significant over control † Significant over previous time point

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control

Fig:3.8. **Activities of aspartate amino transferase in a) serum and b) liver of normal and thioacetamide treated rats at different time points**

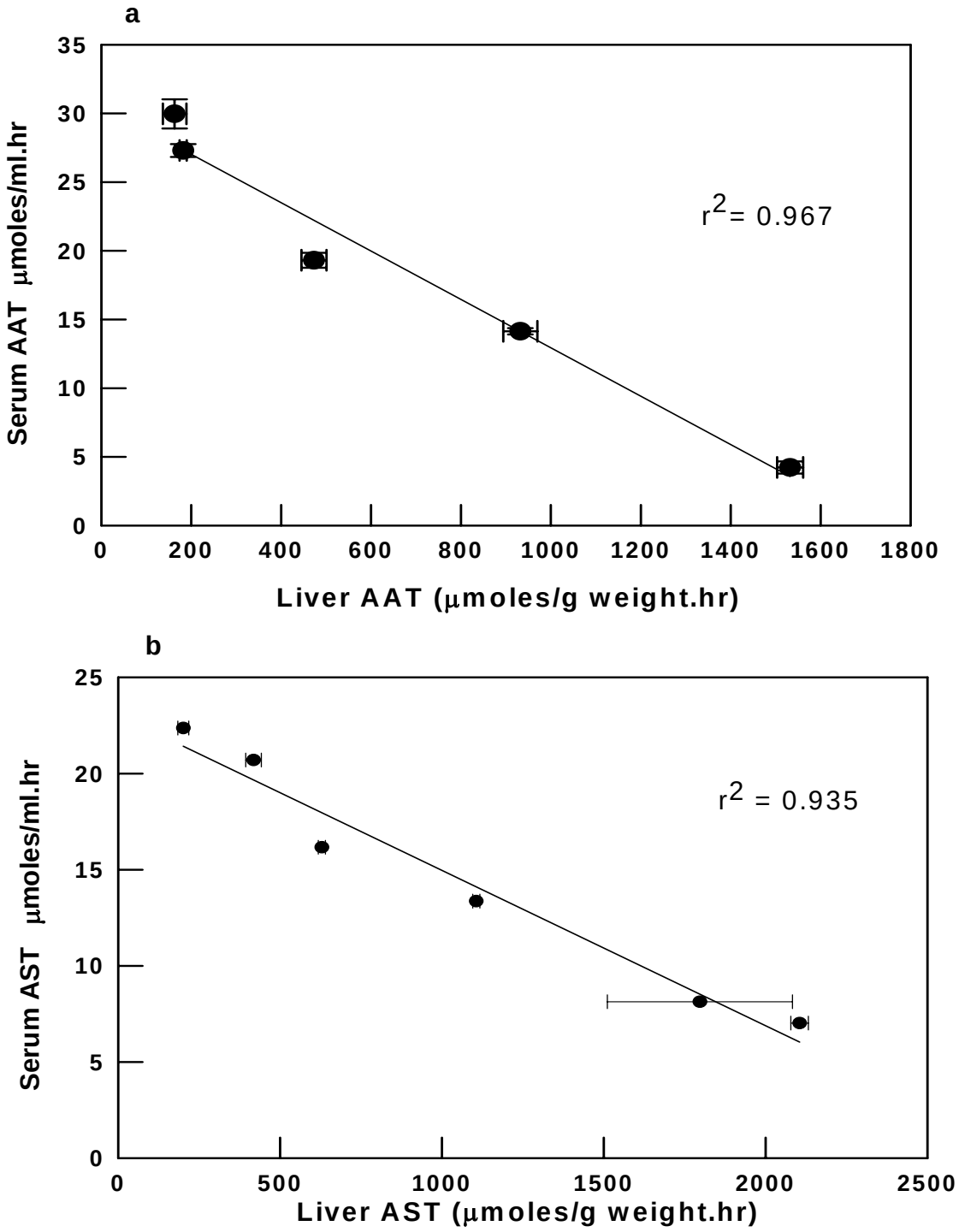


* Significant over control

† Significant over previous time point

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Fig: 3.9 **Correlation between serum and liver alanine aminotransferases (a) and aspartate aminotransferases (b) in thioacetamide treated rats**

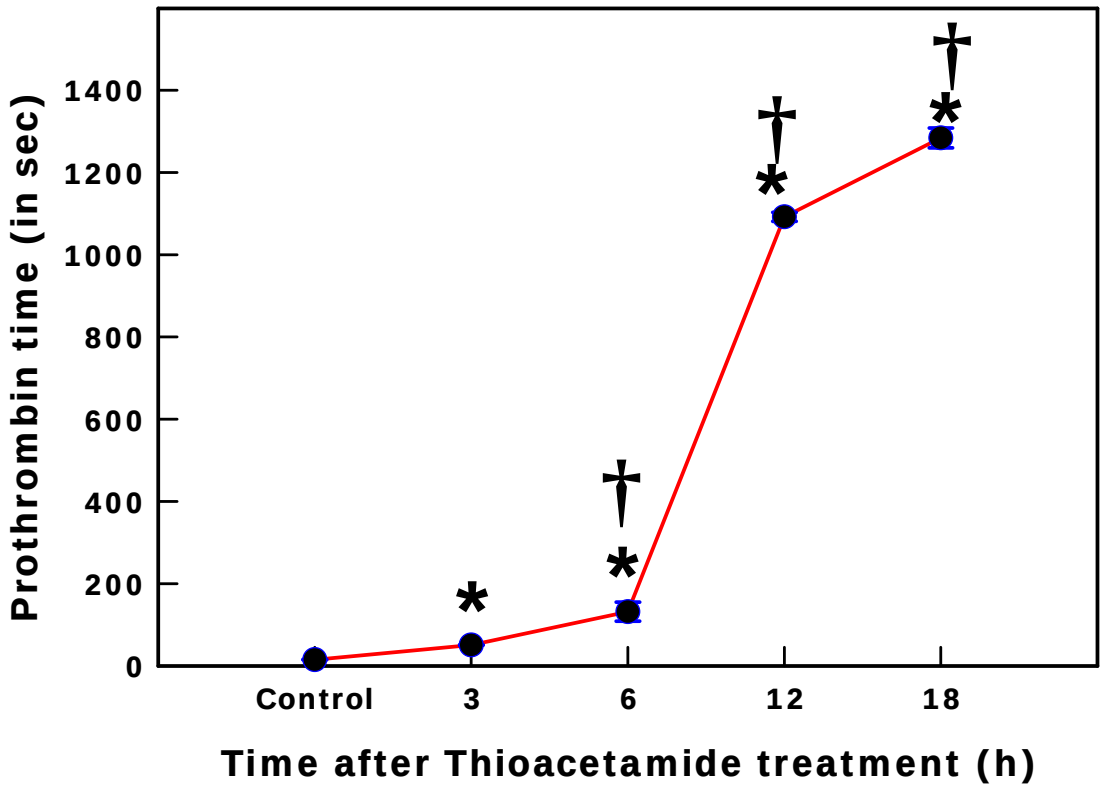


3.2.1.6 Prothrombin Time

During the standardization of the animal model, it was observed that the time required for the clotting of the blood was increasing with 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 24h (Fig 3.10). A three fold increase in PT as early as 6h after the administration of thioacetamide and by 9 fold at the end of 12h was observed. The PT was further increased to 17 min and 21 min at 18 and 24h 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^{2+} and thrombin converts fibrinogen to fibrin. 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.

Fig:3.10. Changes in prothrombin time in normal and thioacetamide treated rats at different intervals of time.



* Significant over control † Significant over previous time point

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

In addition to this, albumin and bilirubin levels were also estimated in the present study as they are also good markers for assessing the extent of liver damage. Albumin is a very soluble, globular protein accounting for 70-80% of the colloid osmotic pressure of plasma which is the predominant reason for its clinical use. However, the results of these will be discussed in the following

chapter as it has got more relevance there accounting for the changes observed in tryptophan levels in serum and brain.

The present studies therefore, clearly indicate impaired liver function 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.

3.3 Histopathology

In order to assess the damage caused to the liver by TAA, rats were sacrificed at the following time periods (0, 6, 12, 18 & 24h) after the second dose of administration of the drug. The liver specimen was fixed in Bouin's fluid, embedded in paraffin wax, sliced into thin sections, stained the sections with hematoxylin and eosin and observed under light microscopy. The results of the liver histology in the TAA-induced fulminant hepatic failure rats showed extensive necrosis of liver tissue (Fig 3.11-3.15). The areas of the liver tissue undergoing necrosis were darkly stained possessing condensed nucleus in the drug administered rats, where as the sections of the normal rat liver showed distinct nucleus. Marked degeneration of the liver tissue was observed with increasing time periods after the administration of the drug.

Liver Histopathology

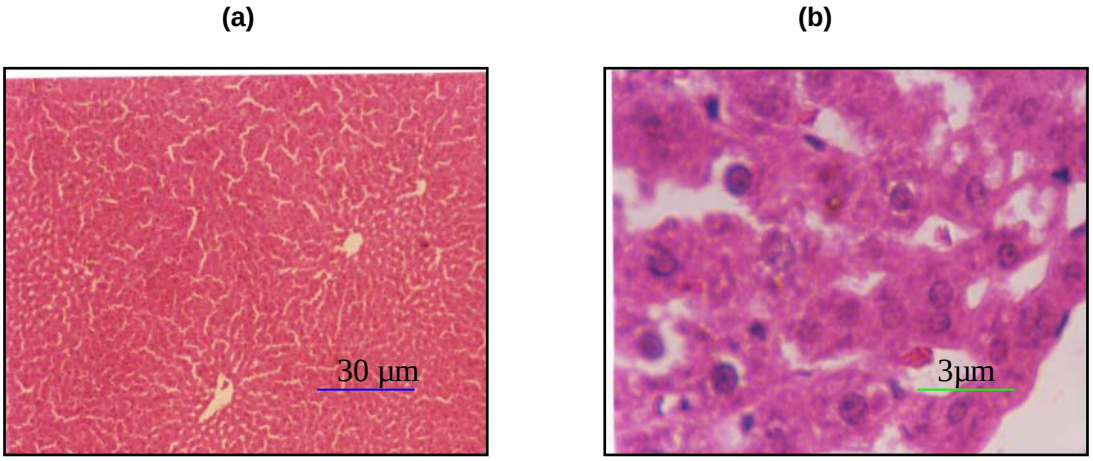


Fig: 3.11 Photo micrographs showing the histological sections of liver cells from the control rats. These cells show the presence of normal hepatocytes with distinct nucleus.

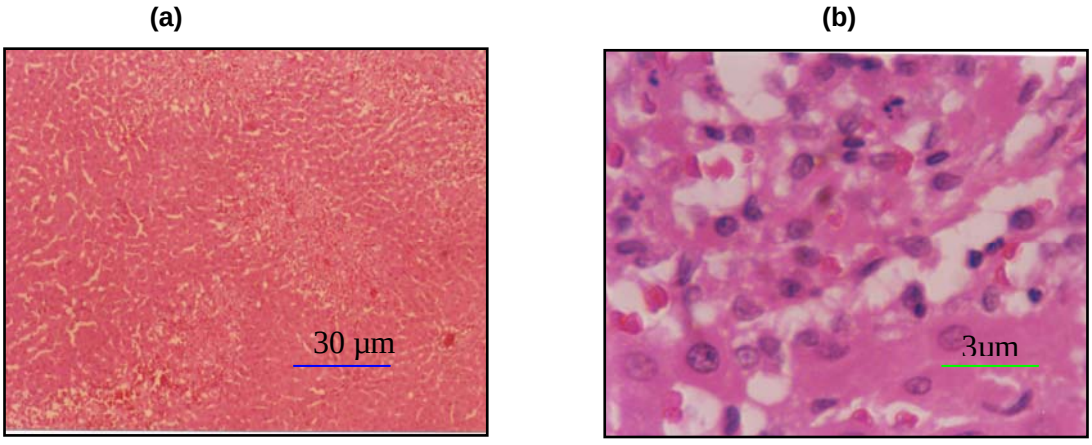


Fig: 3.12 Photomicrographs of liver taken from the rat after 6h of thioacetamide treatment.The micrograph shows the presence of necrotic (degenerating) hepatocytes.

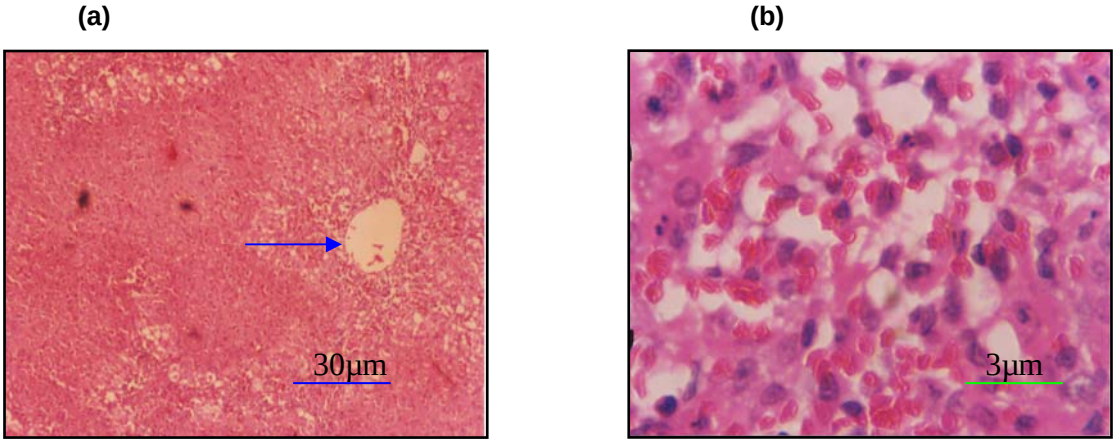


Fig: 3.13 The presence of big patches of degenerating cells with highly condensed nucleus in the hepatocytes were noticed here after 12h TAA administration. These patches are present near the capillary and also away from the capillary indicating the increased area of liver degeneration. Here we can also observe the cytoplasmic shrinkage in the necrotic cells. Few phagocytes were also noticed at this time period of treatment. The changes in the nucleus shape are clearly noticed. Arrow indicate capillary.

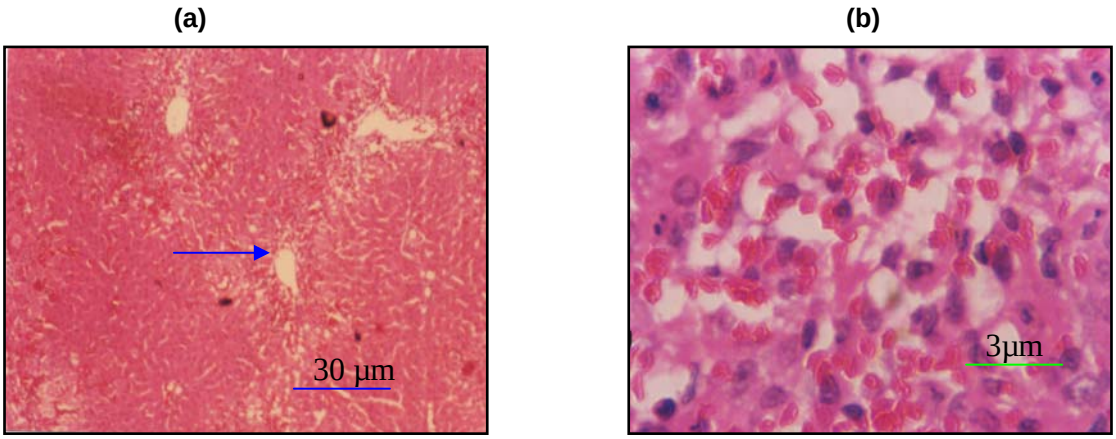


Fig: 3.14 Micrographs showing the effect of thioacetamide at the end of 18 h of treatment. With the longer treatment of thioacetamide there was a significant increase in the degeneration of the liver. Due to the higher degree of necrosis, well condensed nuclei are observed in this preparation. Arrow indicates capillary.

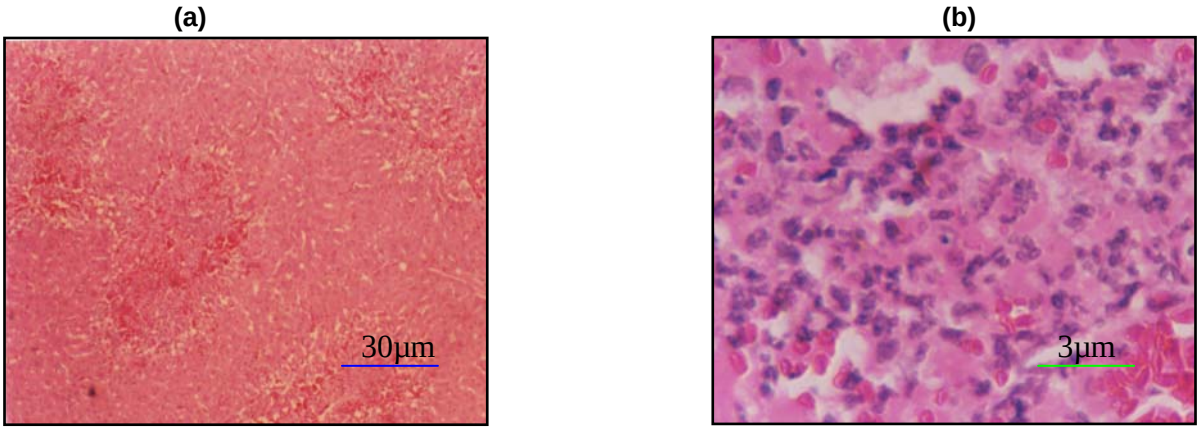


Fig: 3.15 The micrograph here shows the highest degree of damage done to the liver at the end of 24h after the treatment with the drug. Almost all the liver cells are degenerated.

From the present study, it is very clear that TAA induces FHF in 3-months old male Wistar rats. Thus, having ascertained the reproducibility and applicability of this model to the present work, all further studies were restricted to three important brain regions namely Cerebral Cortex (CC), Cerebellum (CE) and Pons Medulla (PM) (Fig 3.16). The reasons for selecting different regions in brain are due to the heterogeneous nature of brain both in structure and function. In addition, all further studies have been carried out at 18h after TAA administration.

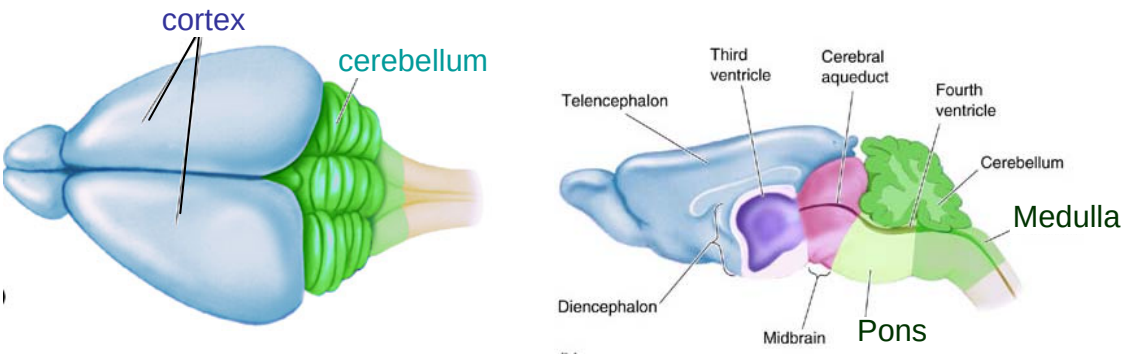


Fig 3.16 Anatomy of rat brain

By 6h as evident from histopathology liver necrosis and other biochemical parameters are just initiated and ammonia levels in serum and brain begin to increase. Hence, studying cerebral parameters before 12h does not reflect any major changes due to hyperammonemia. By 24-36h the animal progresses towards terminal stage and hence studying changes at these intervals may not help clearly differentiate the cause-effect relationship & initial events occurring in brain during HE. By 18h, on the other hand, serum and brain ammonia levels attain substantially high pathological concentrations. Further, maximum increase in serum and brain tryptophan levels was observed at this interval. Hence, this interval was chosen as the ideal one for studying changes in FHF.

Chapter 2
Amino acids

4. Pathogenesis of Thioacetamide induced HE

4.1 Amino acids:

Alterations in amino acid (AA) and protein metabolism are among the prominent metabolic abnormalities seen in patients with both chronic and acute liver failure. Impaired production of albumin and clotting factors are all related to alterations in AA and protein metabolism (Herrmann and McIntyre 1991; Jones and Gammal, 1988), common in liver dysfunction. Consequently the role of the liver in AA metabolism has received much attention and characteristic alterations in the profile of circulating amino acids have been reported in a variety of acute and chronic liver diseases (Wu et al., 1955; Iber et al., 1957). Study of AA metabolism assumes significance in context of liver dysfunction, as altered amino acid profiles in liver and thereby in serum may influence cerebral amino acid metabolism in adverse ways.

Although consistent findings with respect to increase in aromatic amino acids in serum were observed in different models of HE, the same was not true with regard to other amino acids specially the branched chain amino acids (BCAA). These discrepancies observed might be attributed to differences in animal models, toxins used, dosage and other treatment parameters. The elevation of aromatic amino acids (AAA) in liver disease is primarily thought to reflect impaired hepatic clearance, while the changes in the BCAA is attributed to changes in muscular metabolism. However, the pathophysiology of these alterations are still poorly understood (Hellerstein and Munro, 1988). The findings of the increased AAA levels and decreased BCAA levels in serum led Fischer

and Baldessarini (1971), to propose the so called “false neurotransmitter” hypothesis as both AAA and BCAA share the same carrier mediated transport mechanism into brain. Reduced competition from BCAA at the blood- brain barrier allows increased flux of AAA into the brain. Saturation of the enzyme tyrosine hydroxylase, which normally converts tyrosine to dopa leads to preferential decarboxylation of tyrosine to tyramine, followed by β-hydroxylation to the ‘false neurotransmitter’ octopamine. By a similar mechanism phenylalanine can be converted to β-phenylethanolamine. Altered cerebral amino acid metabolism in HE may also result in impaired amino acid-ergic neurotransmission giving rise to observed clinical deterioration. Increase in tryptophan levels lead to alterations in the neurotransmitter serotonin which is associated with a wide range of physiological functions like sleep, mood and circadian rhythms. Increase in the tryptophan levels could also lead to increased catabolism via the kynurenine pathway which leads to the formation of neurotoxins like 3-hydroxykunurenine and quinolinic acid.

With this view of the potential adverse effects of altered amino acid metabolism on cerebral functions, the present study was aimed to investigate changes in amino acid profiles of serum and brain following thioacetamide induced liver failure.

4.1.1 Results:

Changes in amino acid profiles of Serum and Brain:

Levels of tryptophan, phenylalanine, tyrosine, glutamine, proline, and alanine increased significantly in serum and all regions of brain studied viz., CC, CE & PM while the BCAA did not show any particular pattern in both serum and brain (tables 4.1-4.4).

Table 4.1 Levels of amino acids in the cortex of normal and TAA treated rats.

Serial number	Amino acids	Normal nmoles/g wet weight	TAA treated (18h) nmoles/g wet weight	Percent change
1	Tyrosine	100±8.8	150.66±3.5	49.3*
2	Methionine	49±1.5	49.33±4.6	No change
3	Valine	114.3±9.06	114±8.08	No change
4	Leucine	66±3.5	116.33±9.33	+76*
5	Isoleucine	39.66±5.3	48±10.8	+23
6	Phenylalanine	30 ±1.15	50.6±6.17	+69*
7	Tryptophan	24.66±2.6	62.33±4.4	+153*
8	Aspartic acid	2400±57.73	1866±33.3	-22*
9	Glycine	980±24.3	1200±57.7	+22.4*
10	Glutamic acid	14666±742.37	12800±400	-13
11	Arginine	140±6.6	103±5.7	-26*
12	Alanine	573.7±15.4	867.3±26.2	+51.3*
13	Glutamine	12800±35.3	19500±30.5	+52.3*
14	Proline	174.3±3.28	213.3+3.38	+22.3*

Table 4.2 Levels of amino acids in the cerebellum of normal and TAA treated rats.

Serial number	Amino acids	Normal nmoles/g wet weight	TAA treated (18h) nmoles/g wet weight	Percent change
1	Tyrosine	108±5.54	145±6.37	+34*
2	Methionine	56.33±4.33	74±7.50	+31
3	Valine	88.66±6.22	58.66±13.42	-34
4	Leucine	63.33±8.4	102.66±6.34	+62*
5	Isoleucine	36.33±2.78	50.33±11.2	+39
6	Phenylalanine	21.33±0.33	51±1.5	+143*
7	Tryptophan	20.33±1.45	50.33±3.52	+148*
8	Aspartic acid	2450±28.86	1900±57.73	-22*
9	Glycine	1033±88.19	1200±200	+16
10	Glutamic acid	9866±325.9	11276±448.4	+14
11	Arginine	146.66±8.19	100±5.73	-31.5*
12	Alanine	503±11.93	1020±75.71	+102*
13	Glutamine	8280±436	15250±106	+84*
14	Proline	131±15.1	193.66±11.2	+48*

Values are Mean ± S.E.M, n=3. Statistical analysis was done using one way ANOVA with Student Newman-Keuls test. Level of significance was set at $P< 0.05$.* Significant over control.

Table4.3. Levels of amino acids in the Pons Medulla of normal and TAA treated rats.

Serial number	Amino acids	Normal nmoles/g wet weight	TAA treated (18h) nmoles/ g wet weight	Percent change
1	Tyrosine	104.66±6.22	151.66±4.41	+45*
2	Methionine	52±9.45	65.66±4.3	+26
3	Valine	94.3±1.45	79.3±0.88	-16*
4	Leucine	70.66±6.06	93.33±7.055	+32
5	Isoleucine	45.33±1.45	76±1.15	+69*
6	Phenylalanine	20.12±2.0	43.33±4.4	+115*
7	Tryptophan	21.66±1.33	59.66±6.0	+175*
8	Aspartic acid	2466±88.2	1833±120	-26*
9	Glycine	1213.7±57.9	1300±26.5	+7
10	Glutamic acid	8433.33±717.2	6000±230	-29*
11	Arginine	189±17.32	130.7±3.3	-31*
12	Alanine	456.7±23.3	893.3±13.3	+96*
13	Glutamine	10000±179	16200±132	+62*
14	Proline	106.7±4.18	151.3±3.76	+42*

Table 4.4. Levels of amino acids in serum of normal and TAA treated rats.

Serial number	Amino acids	Normal nmoles/ml	TAA treated (18h) nmoles/ml	Percent change
1	Tyrosine	92.33±5.81	130±5.2	+41*
2	Methionine	58.3±0.5	67.1±5.6	+15
3	Valine	184±6.1	216.3±1.9	+18*
4	Leucine	163.3±4.33	169.3±1.8	+4
5	Isoleucine	122±4.7	101±8.6	-17
6	Phenylalanine	65.33±9.2	108.66±1.45	+66*
7	Tryptophan	97.6±2.9	155.3±9.2	+59*
8	Aspartic acid	21.1±8.04	47.6±4.9	+126*
9	Glycine	269.4±11	551±17	+105*
10	Glutamic acid	275.2±0.9	90±0.6	-67*
11	Arginine	102.7±6.33	93±3.21	-9
12	Alanine	97±2.8	162.7±1.45	+68*
13	Glutamine	610±8	1390±13	+127
14	Proline	97.3±5.81	136±5.29	+38

Values are Mean ± S.E.M, n=3. Statistical analysis was done using one way ANOVA with Student Newman-Keuls test. Level of significance was set at $P< 0.05$.* Significant over control.

Representative HPLC chromatograms of the standard amino acids and their levels in the serum of normal and TAA treated rats.

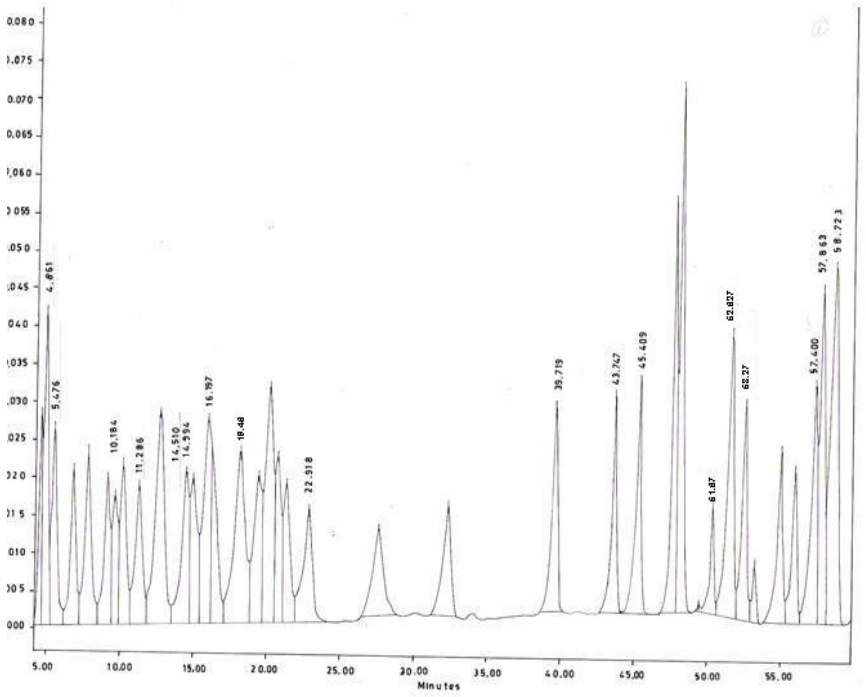
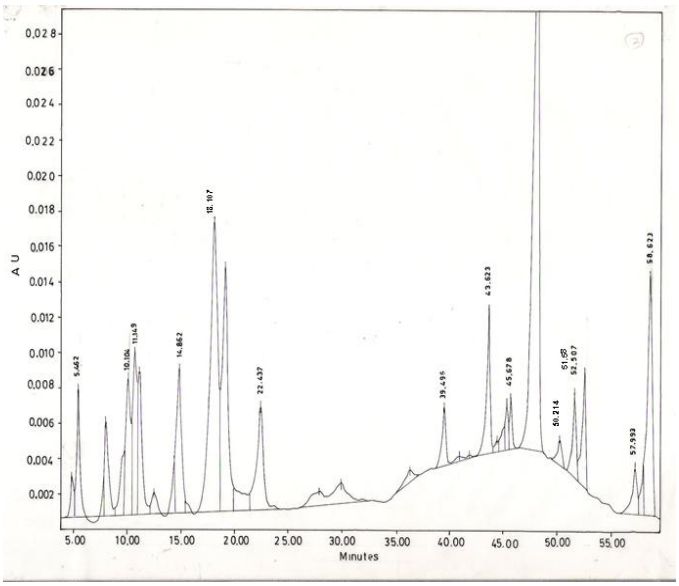


Fig:4.1.1 Profile of standard amino acids separated on a C_{18} reverse phase column (250 X 4.6 mm) on HPLC using photo-diode array detector with wavelength set at 254nm. The conditions used are described in the materials and methods section. The retention times of each standard is as follows: Aspartic acid- 4.86,Glutamic acid-5.47,Glycine- 10.8, glutamine-11.28, Arginine-14.19, Alanine-19.46, Proline -22.19, Tyrosine-39.71, Valine- 43.74, Methionine -45.40, Isoleucine-51.67, Leucine-52.6, Phenyl alanine-57.66 and Tryptophan-58.72 mins.

A.



B.

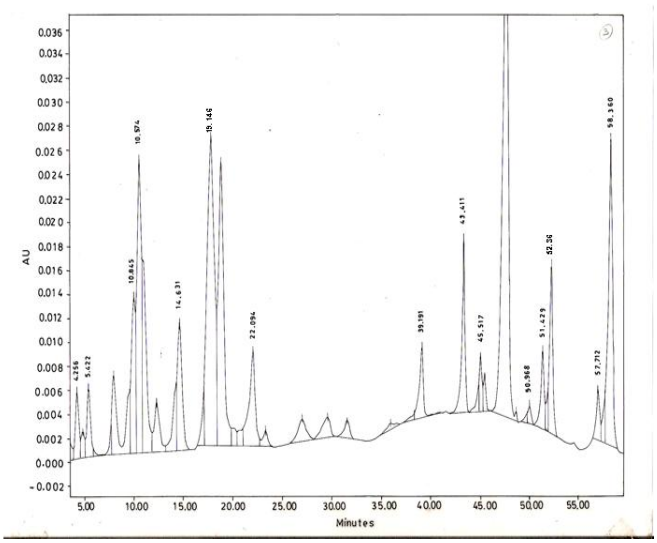


Fig: 4.1.2 **HPLC** profile of serum amino acids in normal (A) and TAA treated (B) rats. A marked and significant increase in tryptophan was observed in TAA treated rats when compared to control. Quantification was performed by peak area analysis.

4.1.2 DISCUSSION:

The increase in the levels of most of the AA in serum during TAA induced FHF could be due to an increased influx from extra hepatic tissues by protein degradation and/or decreased gluconeogenesis and protein synthesis in liver. In addition, the significant increase could also be attributed to the leakage from the liver reflecting severe necrosis of the tissue. Such increase in most of the amino acids in serum was reported by Record et al. (1976). The increase in brain levels of amino acids might reflect endogenous alterations in cerebral amino acid metabolism or could only be secondary to the increase in serum levels, suggesting an increased influx into the brain via carrier mediated amino acid transport and / or a partial blood brain barrier damage as shown in (Fig 4.5.3). The amino acids that most commonly interfere with brain functions are large AAA including tryptophan, phenylalanine and tyrosine. Whether they would alter the excitability potential by forming excitotoxins is therefore worth studying.

The current study indicates increase in all the AAA in different regions of brain suggesting altered neurotransmission. Several others reported a similar increase in AAA during HE (Curzon et al., 1973, Cummings et al., 1976; Mans et al., 1979). Alterations in these AAA would have gross consequences as they form important neurotransmitters in brain. For example, excess tryptophan leads to the formation of increased inhibitory neurotransmitter serotonin apart from other neurotoxins while excess of phenylalanine and tyrosine would lead to the formation of false neurotransmitters.

Other consistent findings in different models of acute liver failure are increase in levels of brain ammonia and glutamine and a decrease in aspartate, glutamate or both (Biebuyck et al., 1974; Record et al., 1976; Hindfelt et al., 1977; Bosman et al., 1990; Dejong et al., 1992). However, glutamate levels in the present study showed a slight increase in cerebellum, though insignificant. The increase in glutamine levels in brain, therefore, may be the result of increased detoxification of ammonia via glutamine synthetase. Glutamine is one of the important osmolytes and can cause edema (Albrecht and Dolinska, 2001). Several studies have pointed to a good correlation between the elevation of brain glutamine and edema in acute hyperammonemic animals (Swain et al., 1992, Blei et al., 1993). Astrocytes are the loci of glutamine synthesis in the central nervous system (Norenberg and Martinez-Hernandez, 1979) and at the same time the primary site of ammonia –induced water accumulation *in vivo* (Traber et al., 1987). The marked swelling of the astrocytes observed in the histological sections of brain (Fig 4.2.1) supports such a possibility. Ultra structural observations of the astrocytes also revealed increased cytoplasmic swelling, mitochondrial swelling and nuclear condensation (Fig 4.2.2). Increased glutamine levels in brain may also contribute to increased tryptophan accumulation in brain by accelerated exchange (Albrecht and Dolinska, 2001) Other factors involved in tryptophan accumulation include increased unidirectional transport by the L-system (Cardelli-Cangiano et al., 1981) and activation of cerebrovascular gamma glutamyl transpeptidase (Gorgiewski-Hriosho et al., 1986). Though glutamine has been considered to be non toxic as

it does not excite neurons (Bradford and Mcllwain, 1966), nevertheless, it has a substantial role in the pathophysiology of HE. Arginine levels decreased significantly in all the regions of brain and are in accordance with Mans et al. (1994). This is of significance as arginine is the substrate for nitric oxide synthase through out the brain and the body (Mans et al., 1994) and nitric oxide synthase mediated oxidative damage is implicated in Hepatic encephalopathy (Norenberg, 2003). This may indicate increased breakdown of arginine through NOS. Among the branched chain amino acids, valine increased in serum while isoleucine and leucine did not show any statistically significant change (Table 4.4). In brain, valine decreased and isoleucine (Tables 4.1-4.3) increased significantly only in pons medulla while in cortex, valine and isoleucine did not show any significant change. In cerebellum a decrease in valine and a slight increase in isoleucine were observed, but not statistically significant. Leucine, on the other hand, increased significantly only in cortex and cerebellum regions while the increase in pons medulla was not statistically significant. Several controversies exist regarding the role of branched chain amino acids in serum with some reporting either an increase (Mans *et al*., 1979) or decrease (Knell *et al*., 1974) or no change (Buxton *et al.,* 1974). However, this discrepancy could be attributed to the differences in animal models, toxins used, dosage and other treatment parameters. It is evident from the above studies that TAA-induced FHF results in alterations in the amino acid profiles of both serum and different regions of brain. The foregoing studies indicate enhanced accumulation of certain cytotoxic amino acids in different regions of brain suggesting possible ultrastructural changes

leading to impairment of blood-brain barrier. Hence, it would be interesting to probe further into ultrastructural changes in the brain resulting out of TAA-induced HE. As some of the tryptophan metabolites are potent lipid peroxidants , neuromodulators and free radical generators, it would be interesting to probe further into tryptophan metabolism.

Tryptophan Metabolism

4.2 Tryptophan metabolism

Analysis of amino acid changes in serum and different regions of brain indicated an increase in aromatic amino acids during HE. Among the aromatic amino acids tryptophan metabolites like serotonin, kynurenic acid and quinolinic acid are known to have neuromodulatory, neuroprotective and neurotoxic effects. Further, some of them are known to induce oxidative stress and have been implicated in most of the neurological disorders like AIDS-Dementia, Huntington's disease, cerebral ischemia, hepatic encephalopathy and Parkinson's disease (Berqvist et al., 1995; Michalak et al., 2001; Pearson et al., 1992; Saito et al., 1993; Stone et al., 2003). The essential amino acid tryptophan can be metabolized via two important pathways in the brain:

1. **The Indolamine pathway**, to produce the neuroactive metabolites serotonin (influencing mood/depression) and melatonin (effecting sleep patterns) as shown in figure (Fig 4.2.1) and

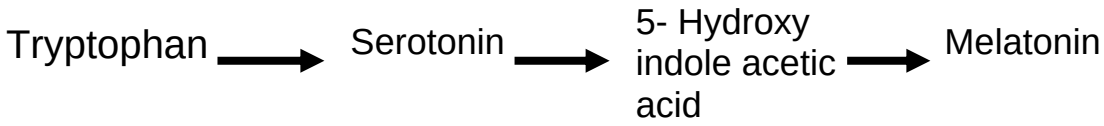


Fig: 4.2.1 Schematic flowchart showing the indoleamine pathway, via the serotonin, leading to the formation of melatonin.

2. **The Kynurenine pathway**, from which a number of neurotoxic metabolites are produced, some of which have excitatory activity. The final product of tryptophan catabolism via the kynurenine pathway is the electron transporter, Nicotinamide Adenine Dinucleotide (NAD), which is essential for oxidative

phosphorylation (energy production) in the cell (Fig 4.2.2). Kynurenines have attracted the interest of neuroscientists because of their possible role in the pathogenesis of human neurodegenerative diseases (Freese et al., 1990). In particular, abundance of the endogenous excitotoxin, quinolinic acid, was shown to be associated with pathological conditions (Stone, 2001a). Also the hypofunction of kynurenic acid, a broad spectrum antagonist of excitatory amino acid receptors, has been hypothetically linked to the occurrence of seizures and cell death (Harris et al., 1998).

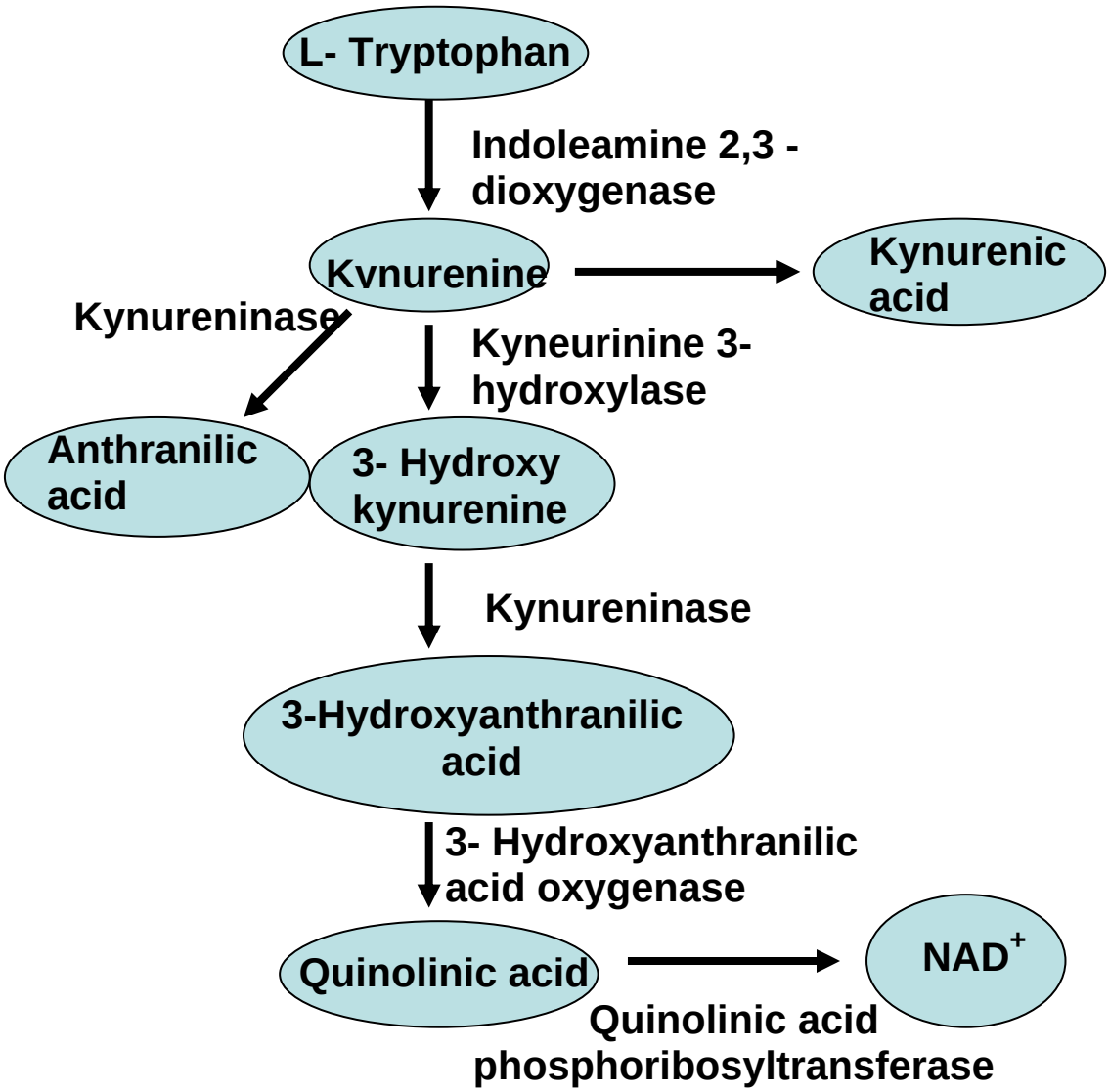


Fig: 4.2.2 Schematic representation of tryptophan metabolism via the Kynurenine pathway.

In brain, serotonin biosynthesis depends on the quantity of tryptophan which crosses the blood-brain barrier. Only free plasma tryptophan, i.e., unbound to albumin, penetrates into the brain. Decrease in unbound tryptophan reduces its penetration. Moreover, other large neutral amino acids are in competition with

free tryptophan and limit its entry into the brain. Albumin, which is synthesized and secreted by liver into blood, carries out several functions in the blood. It binds to several compounds and xenobiotics such as drugs and transports into tissues. Of the several compounds bound by albumin, free fatty acids, tryptophan and bilirubin assume importance in FHF. These compounds bind at the same site on albumin. Decrease in albumin levels has significant implications on tryptophan transport as less amount of tryptophan will be bound to the albumin. This would increase the free tryptophan pool in the blood. This free pool of tryptophan is known to be the precursor for the synthesis of neurotransmitter, serotonin, in brain and quinolinic acid via the kynurenine pathway. Tryptophan being an essential amino acid, all tryptophan in brain is obtained through blood. Though alterations in the serotonin pathway metabolites have been implicated in HE in patients and experimental models, it is still unknown whether HE is associated with functional alteration of central serotonergic neurotransmission or not (Herneth et al., 1998). Moreover, not much work has been reported in the kynurenine pathway metabolites in Hepatic encephalopathy. Basile et al., (1995) has reported an increase in the quinolinic acid at the end stages of HE. However, not much work has been done with respect to other kynurenine metabolites. Moreover, alterations in these pathways vary based on the type of model, dosage and the drug used. In the present study also tryptophan levels were elevated in the serum and brain tissues of animals treated with TAA, which could result in alterations in the tryptophan metabolites. Hence the present study is

aimed to investigate changes in the level of various tryptophan metabolites in different regions of brain during HE.

4.2.1 Results:

Serum bilirubin (Fig 4.2.3A) and free fatty acids (Fig 4.2.5A) levels increased while albumin levels (Fig4.2.3 B & Fig 4.2.4) decreased significantly during TAA-induced FHF. The decrease in the albumin levels would increase the serum levels of free tryptophan. This was confirmed by significant increase in serum free tryptophan levels (Fig 4.2.5B). Total tryptophan levels (Fig 4.2.5C) also increased significantly in serum, though the extent of increase was less compared to that of free tryptophan levels.

The TAA-induced changes in brain Trp, 5-HT and 5-HIAA are summarized in (Fig 4.2.6A, 4.2.6B & 4.2.6C). These results demonstrate that TAA-induced liver failure was associated with remarkable increase in the cerebral levels of tryptophan though the extent of increase varied in different regions. Increase in 5-HT levels was more or less uniform (~20-30%) in all regions of the brain. 5-HIAA changes were more pronounced in the pons medulla (67%), followed by cortex (52%) and cerebellum (34%).

Kynurenine levels (Fig 4.2.7B) in TAA-induced acute liver failure increased in pons medulla but a slight insignificant decrease was observed in cortex and cerebellum. However, in serum, significant increase was observed in kynurenine levels (Fig 4.2.7 A). The activity of enzyme indoleamine 2, 3

dioxygenase, the first and rate limiting enzyme in tryptophan pathway (Fig 4.2.8) showed similar changes in different regions of brain. Levels of 3-hydroxy kynurenine (Fig 4.2.9 A) in the brain increased by 58 % in the cortex, followed by 29% in the cerebellum and 28% in the pons medulla region. The levels of kynurenic acid (antagonist to the NMDA receptor), on the other hand, decreased significantly by 34% in the cortex, 21% in the cerebellum and 21% in the pons medulla region (Fig 4.2.9B). Anthranilic acid levels in brain increased significantly in all the regions of brain (Fig 4.2.10). Levels of quinolinic acid, a potent lipid peroxidant, also increased in all the regions of brain under investigation, as observed by Immunohistochemistry (Fig 4.2.16).

Changes in bilirubin and albumin levels in TAA-induced FHF

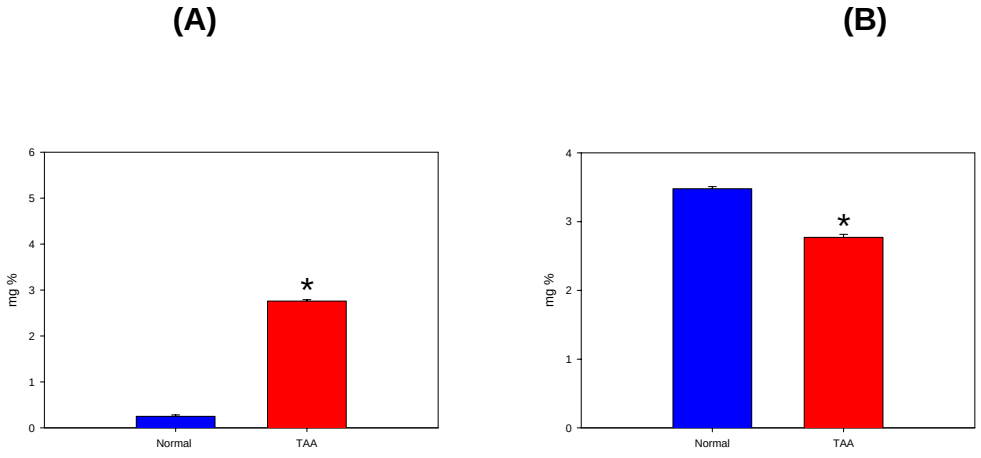


Fig: 4.2.3 Levels of bilirubin (A) significantly increased while albumin (B) decreased significantly in serum. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control**.**

Electrophoretic protein profiles

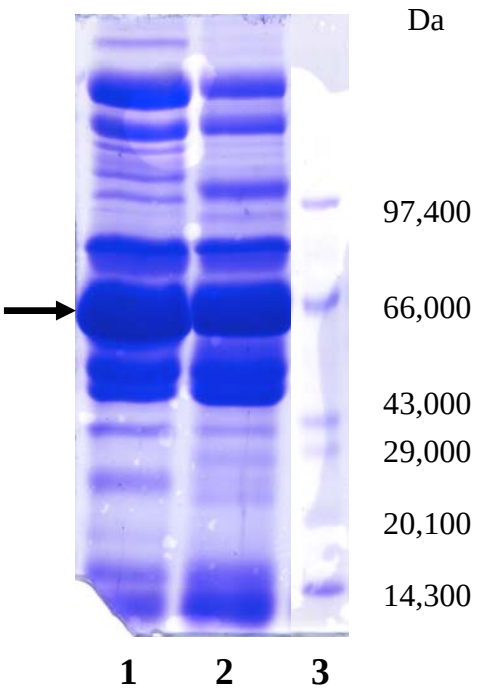
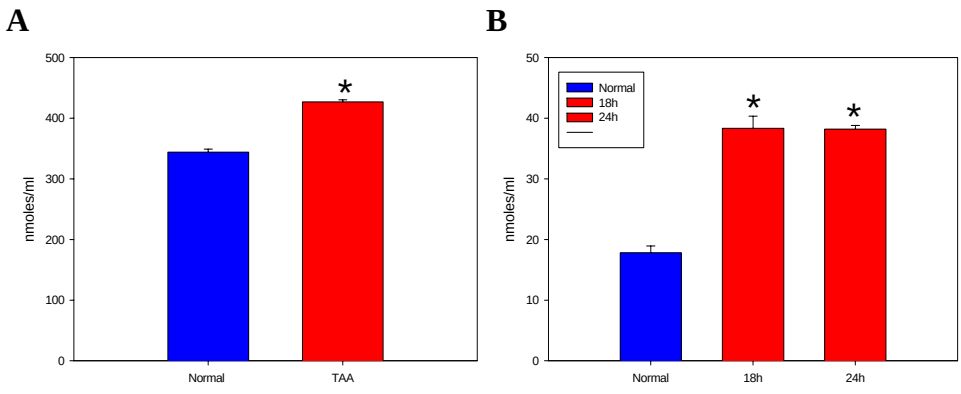


Fig: 4.2.4 Serum protein profile on SDS PAGE gel showing a decrease in albumin (66kDa protein band) levels in TAA treated rats. Lane 1: 100µg of serum protein from normal rat; Lane 2: 100µg of serum protein from TAA treated rat; Lane 3: molecular weight markers.

Changes in serum free fatty acid levels, free and total tryptophan levels in TAA-induced FHF



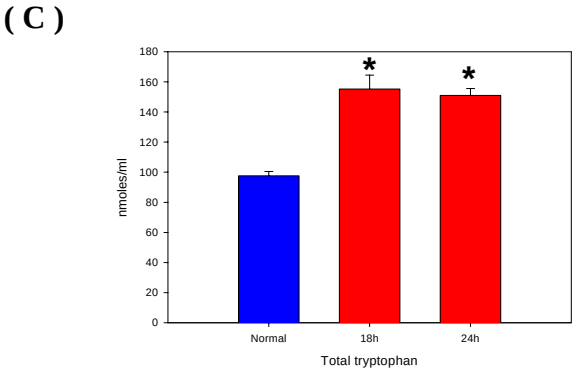


Fig: 4.2.5 Free fatty acids (A), free (B) and total tryptophan (C) levels in serum increased significantly. However, there was no further increase in free tryptophan levels after 18h of TAA administration. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control**.**

Changes in the levels of total tryptophan, Serotonin and 5-hydroxyindoleacetic acid in brain during TAA-induced FHF

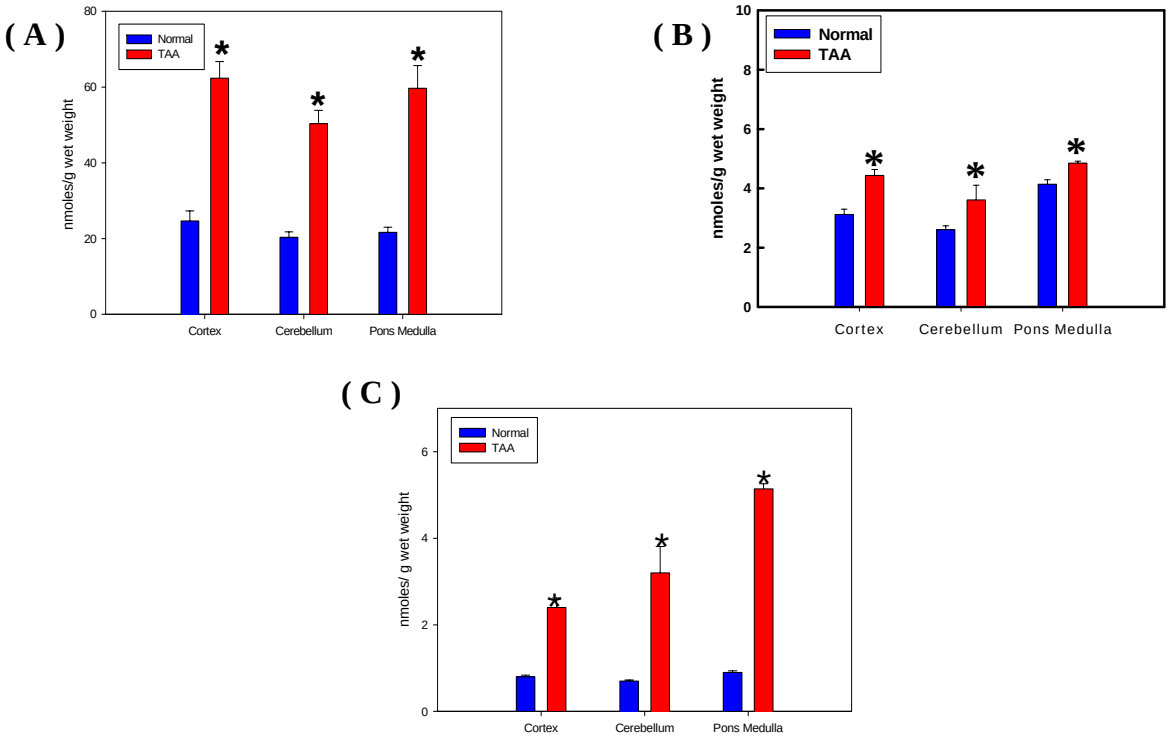


Fig: 4.2.6. Increase in the levels of total tryptophan (A), serotonin (B) and 5-hydroxy indole acetic acid (C) in brain. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control**.**

Tryptophan metabolites:

(A) **(B)**

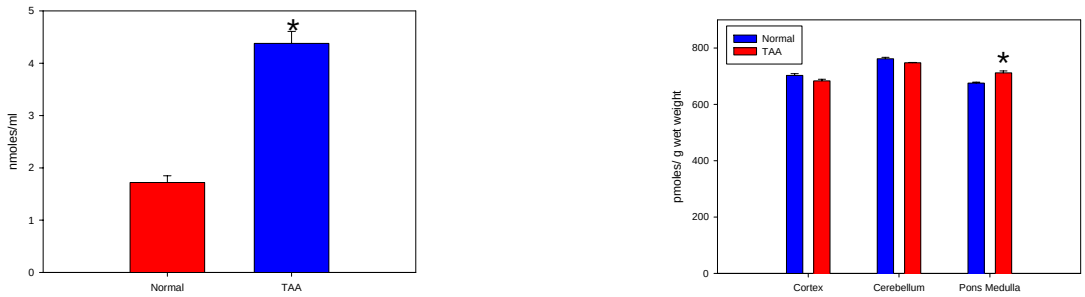


Fig: 4.2.7 Changes in the levels of kynurenine in serum (A) and different regions of brain (B) in both normal and TAA treated rats. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

IDO Activity:

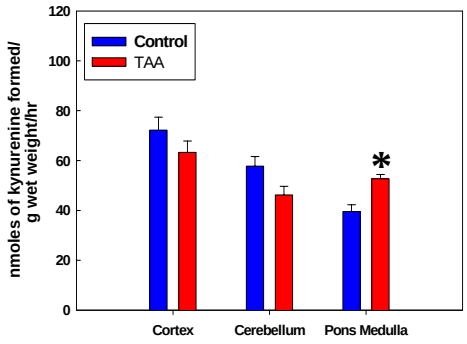


Fig: 4.2.8 Following TAA administration significant increase in the activity of indoleamine2,3 dioxygenase (IDO) was observed only in pons medulla region while there was no significant changes in the other regions. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

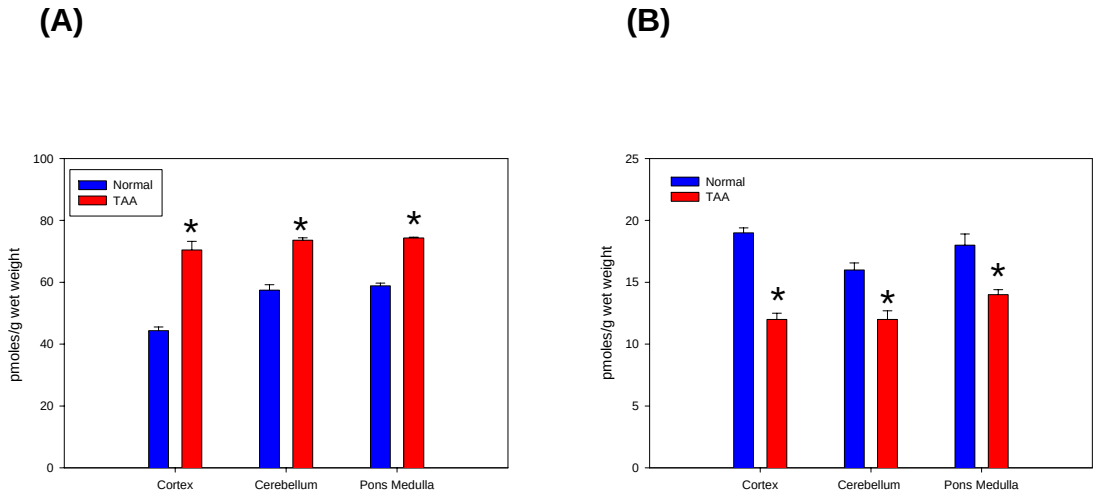


Fig: 4.2.9 3-Hydroxykynurenine levels in brain (A) was significantly increased after TAA treatment and levels of Kynurenic acid (B) decreased significantly in different regions of brain. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

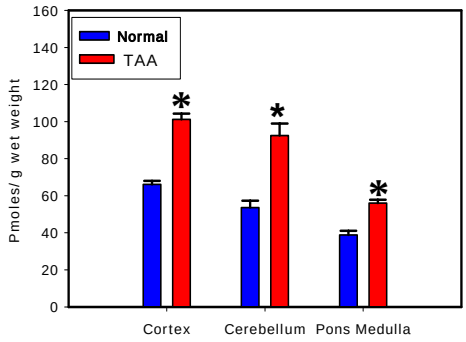


Fig: 4.2.10 Increase in anthranilic acid levels was observed in all the regions of brain in response to TAA administration. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Representative HPLC Chromatogram showing the separation of Trp, 5-HT and 5-HIAA standards and their levels in the cortex region of normal and TAA treated rats.

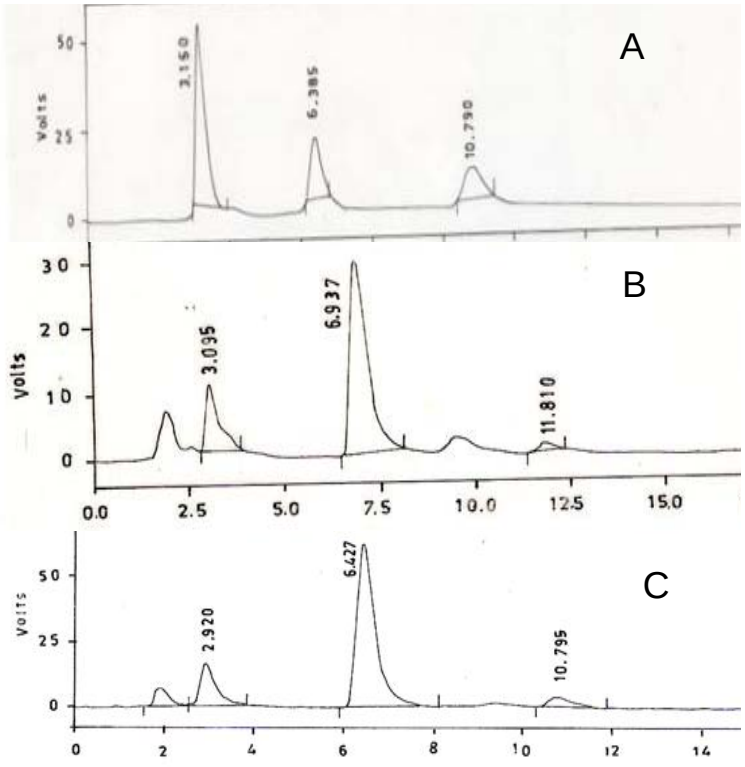


Fig: 4.2.11 A **mixture of standard Trp, 5-HT and 5-HIAA were analyzed by isocratic HPLC on a (RF-10 AXL and a 5µ C_{18} Luna ODS 2 reverse phase column (250 mm X 4.6 mm i. d.) using fluorescence detector with excitation set at 285nm and emission at 345nm. (A). Chromatogram of mixed standard (5-HT, Trp and 5-HIAA retention time, 3.1 min, 6.39 min & 10.79 min respectively). (B) Perchloric extract of a sample of brain from cortex region in normal rats. (C) Perchloric extract of a sample of brain from cortex region in TAA treated rats. The flow rate was 2 ml/minute and the concentrations were quantified based on peak area.**

Representative HPLC chromatogram showing the separation of Kynurenine Standard and its levels in normal and TAA treated pons medulla region.

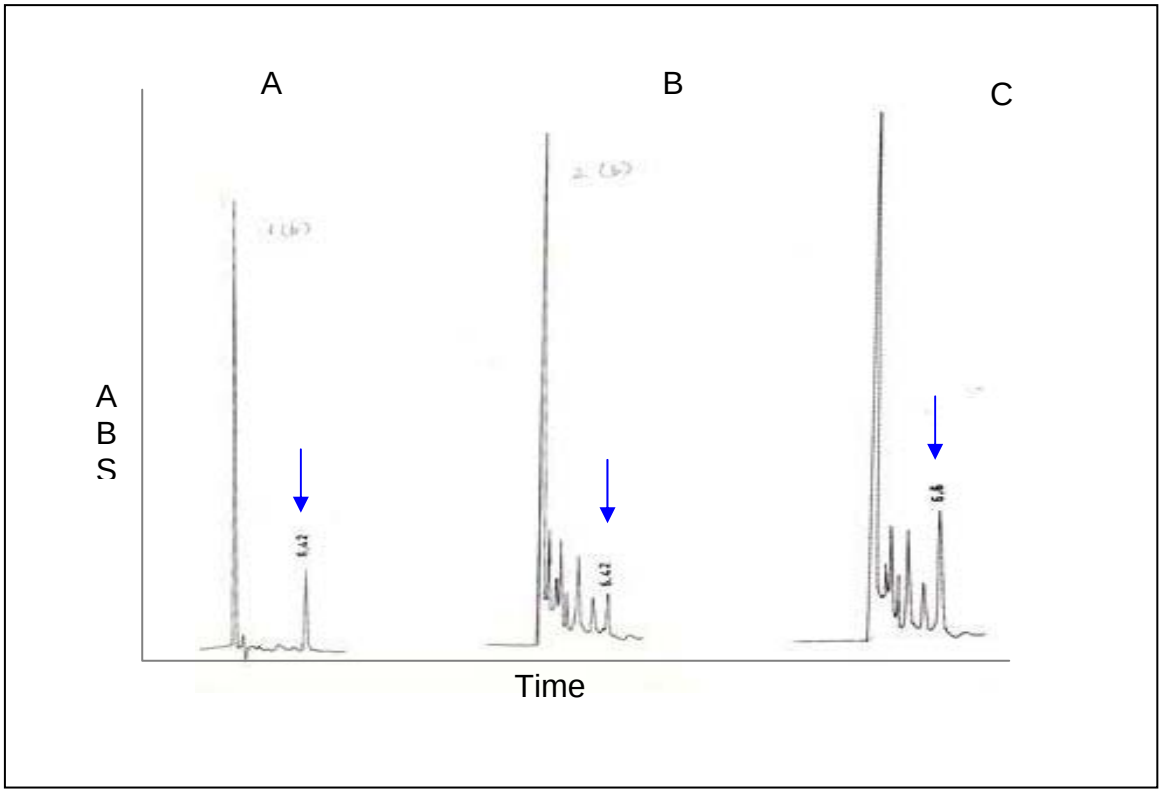


Fig: 4.2.12 HPLC analysis of kynurenine was performed using C_{18} 3.5µ Kromasil reverse phase (150X 3.5 mm i.d.) column with UV detection at 365nm. (A) Chromatogram of Kynurenine standard (retention time 6.4 min), (B) Pons medulla of control rat and (C) Pons medulla of TAA treated rat. The flow rate was 0.5 ml/minute and the concentrations were calculated based on peak area.

Representative HPLC chromatogram showing the separation of 3-hydroxykynurenine Standard and its levels in normal and TAA treated Cerebellum region.

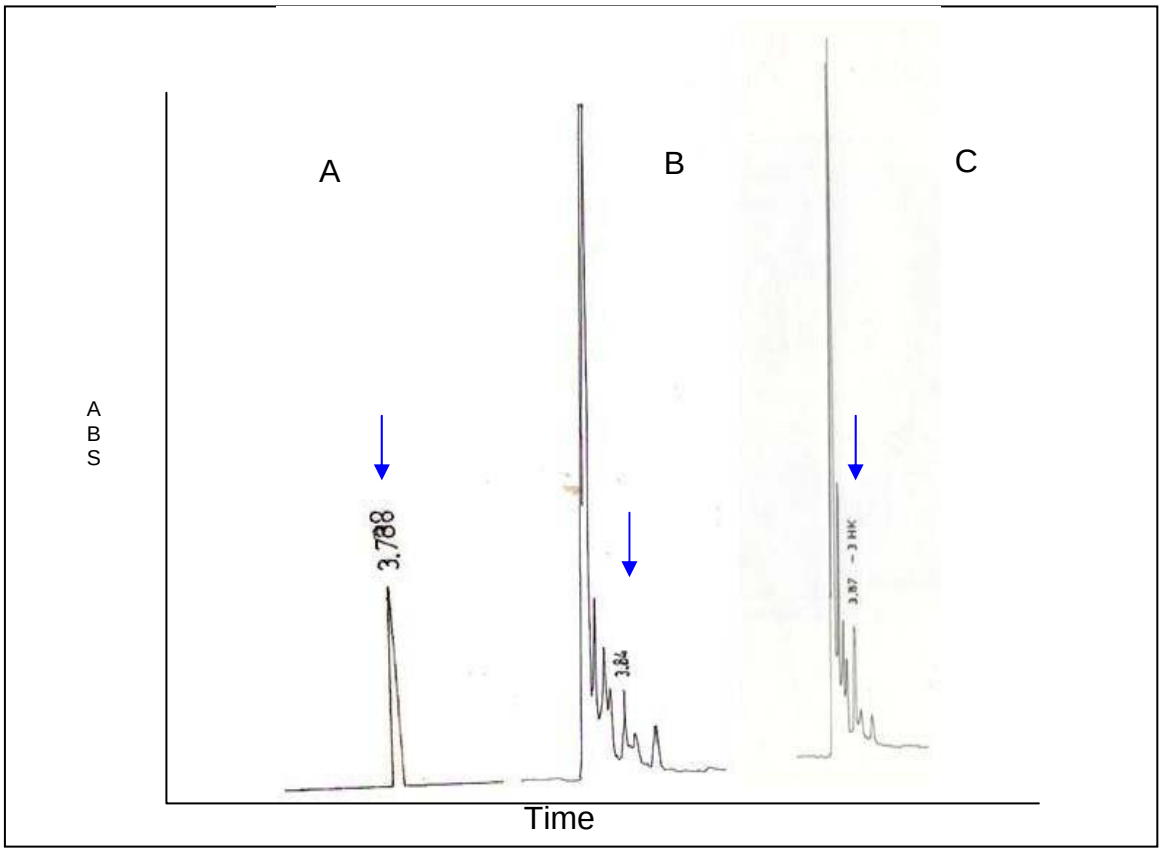


Fig: 4.2.13 HPLC analysis of 3-Hydroxykynurenine was performed using C_{18} 3.5µ Kromasil reverse phase (150 X 3.5 mm i.d.) column with UV detection at 340nm. (A) 3-hydroxykynurenine Standard (retention time 3.7 min), (B) Levels of 3-hydroxykynurenine in the cerebellum of normal rat and (C) 3-hydroxykynurenine levels observed in the cerebellum of TAA treated rat. The flow rate was 0.75 ml/minute and the concentrations were calculated based on peak area.

Representative HPLC chromatogram showing the separation of Kynurenic acid standard and its levels in normal and TAA treated cerebellum region.

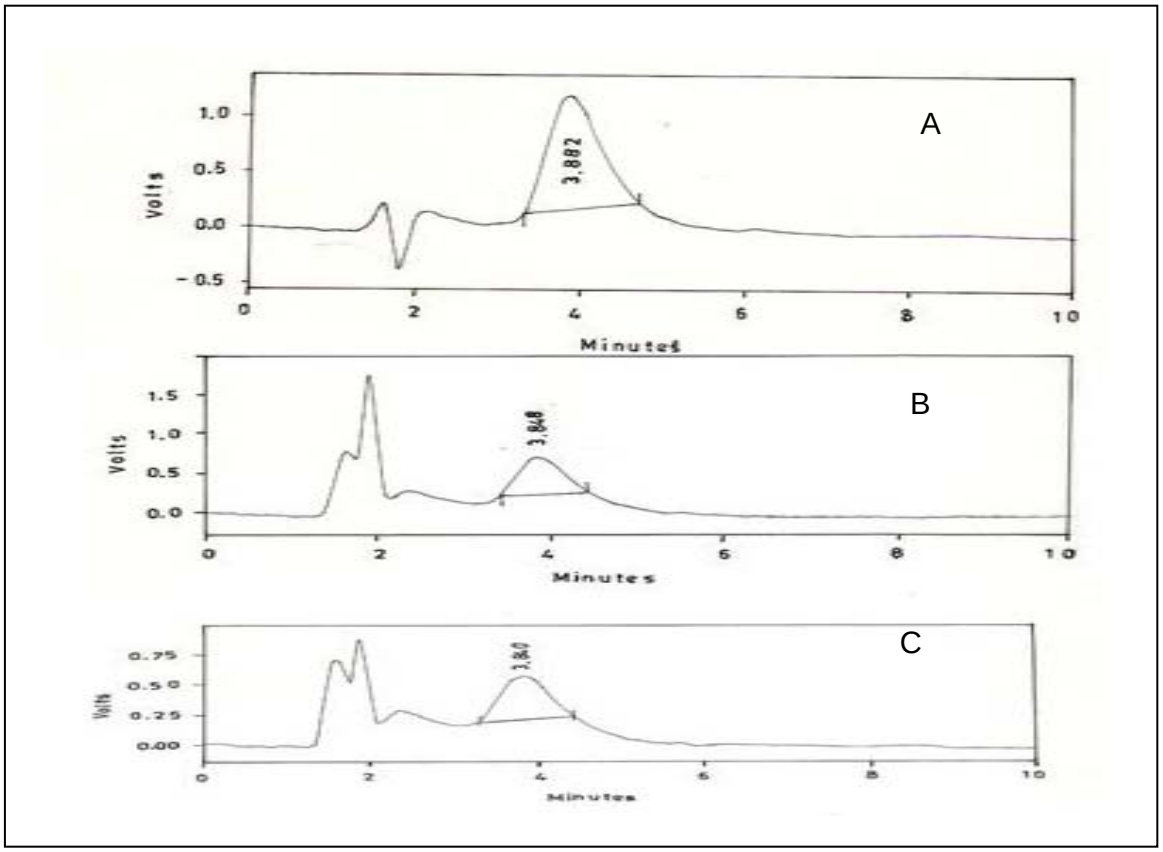


Fig: 4.2.14 HPLC analysis of Kynurenic acid was carried out using 125X 4.6 mm Lichrosphere C_{18} reverse phase HPLC column with fluorescence detector with excitation at 344nm and emission at 398nm. (A) Kynurenic acid Standard (retention time 3.8 min), (B) Levels of Kynurenic acid in the cerebellum region of a normal rat and (C) Decreased levels of kynurenic acid was observed in the cerebellum region of TAA treated rats. The flow rate was 1ml/min and the concentrations were calculated based on the peak area. Note: The scaling is different for all.

Representative HPLC chromatogram showing the separation of Anthranilic acid Standard and its levels in normal and TAA treated Pons medulla region.

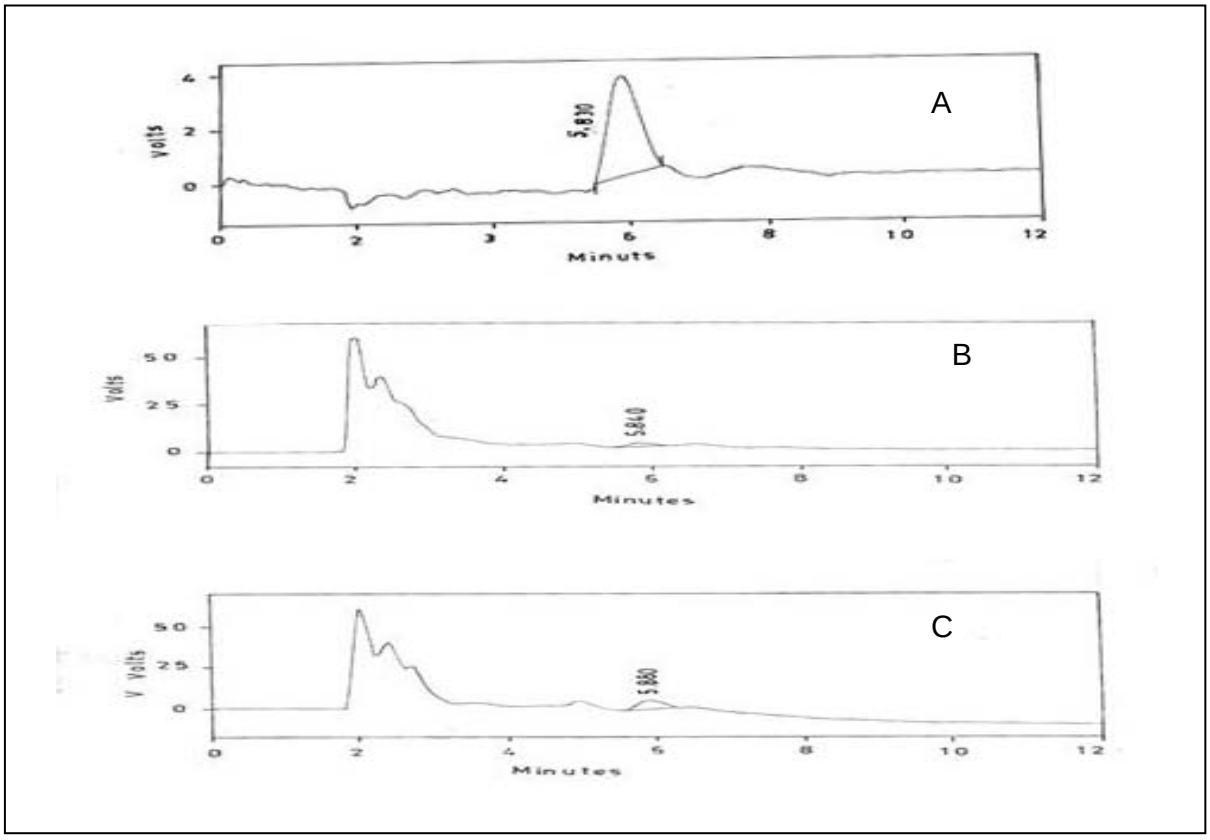


Fig: 4.2.15 HPLC analysis of Anthranilic acid was carried out using 125X 4.6 mm Lichrosphere C_{18} reverse phase HPLC column with fluorescence detector with excitation at 316 nm and emission at 420 nm. (A) anthranilic acid Standard (retention time 5.8 min), (B) Levels of Anthranilic acid in the pons medulla of normal rat and (C) Increased levels of anthranilic acid was observed in the pons medulla of TAA treated rat. The flow rate was 1ml/min and the concentrations were calculated based on the area.

Immunohistochemistry demonstrating increase in quinolinic acid levels following TAA treatment in different regions of brain

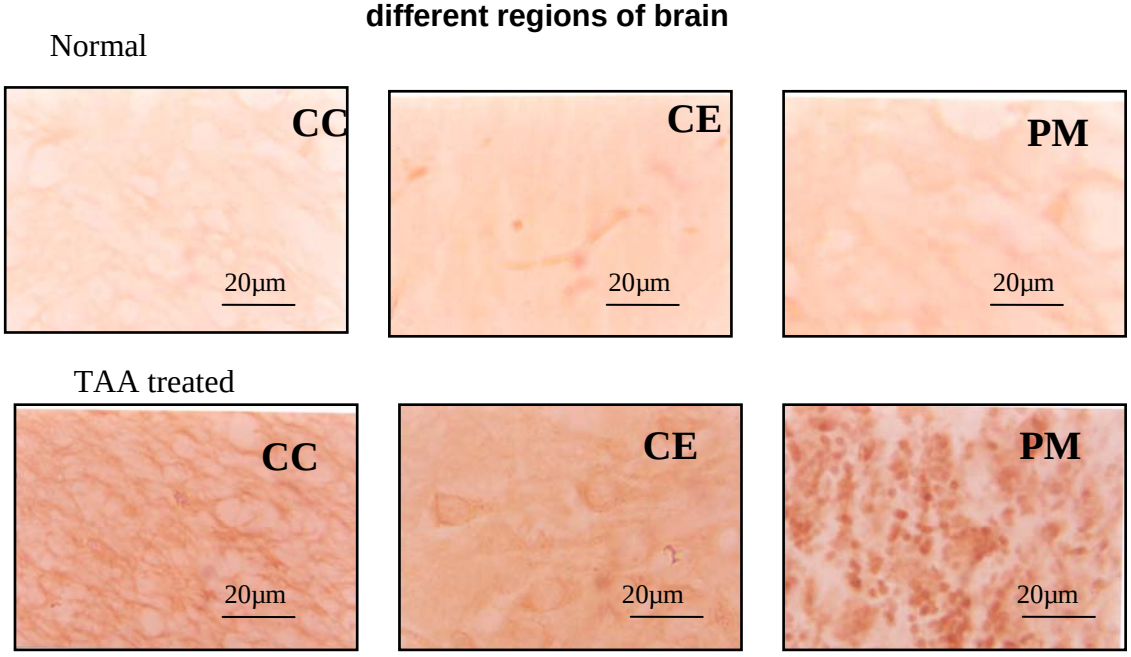


Fig: 4.2.16 Immunohistochemistry using antibodies against quinolinic acid is described under materials and methods. Representative photomicrographs depicting increase in quinolinic acid in different regions of brain in TAA treated rats. The brain section in the normal rats did not show any staining or very minimal when compared to TAA treated rats.

4.2.2 Discussion:

Tryptophan, being an essential amino acid, is not endogenously synthesized in brain tissue and has to be supplied through blood. In the blood, tryptophan exists in two pools-bound and free. The bound pool represents the tryptophan which is non-covalently bound to albumin. The free pool is the tryptophan that is present in the dissolved state in plasma, which is readily available for uptake by tissues including brain. It is interesting to note that the tryptophan binding site on albumin is shared by bilirubin and free fatty acids and hence, there will be a competition between bilirubin, free fatty acids and tryptophan for the same binding site on albumin. Therefore, the amount of tryptophan bound to albumin depends not only on its absolute concentration but also its concentration in relation to other competing factors. The increase in total tryptophan levels in serum is much lower than the increase observed in case of free tryptophan levels. The increase in the bilirubin and free fatty acid levels along with decreased albumin levels could be the most plausible reason for the observed increase in free tryptophan levels in serum and hence more accessibility to the brain. The present finding of elevated tryptophan levels in serum and brain in hepatic encephalopathy also coincides with several other investigators using experimental models and human patients (Ono et al., 1978 ; Jellinger et al., 1978 ; Ferenci 1994 ; Bengtsson et al., 1991 ; Basile et al., 1995). As tryptophan levels increased in brain following TAA treatment, the present work was aimed at studying the alterations occurring in serotonin and

kynurenine pathway, the two alternate pathways of tryptophan metabolism in brain.

Serotonin pathway:

Neurochemical findings in HE are almost exclusively restricted to animal models as human samples are not readily available and even if material is available, postmortem changes would occur due the lapse of time between collection and experimentation. Most of these observations are model dependent and can not be generalized to explain the pathophysiology of HE. However, as mentioned earlier, TAA-induced FHF mimics the human pathological condition to the nearest extent compared to most other models. The results of the present study clearly demonstrate that HE resulting from TAA-induced acute liver failure is accompanied by increased levels of tryptophan in brain. Similar findings were observed by others in cortex during acute liver failure. A uniform increase approximately, 20-30% of serotonin was observed in all the regions of brain after the second dose of TAA administration. This could have important consequences as serotonin is one of the most important neurotransmitter in brain and has a wide array of physiological functions including sleep patterns, circadian rythms, mood disorders and behavior (Adell et al., 1988). However, evidences for the changes in serotonin levels are less- clear cut. In cerebral cortex of pigs after liver ischemia, serotonin levels were found to be unchanged (Curzon et al., 1973; Delorme et al., 1981) while Jonung et al., (1985); Herneth

et al., (1998) ; Yurdaydin et al., (1989) found increased levels of serotonin in rat brain after TAA–induced acute liver failure. Similar findings were observed in animal models of chronic and acute liver failure induced with carbon tetrachloride (Knott and Curzon 1975; Bengtsson et al., 1987). Nevertheless, in the present study, the increase in serotonin levels could be either due to increased synthesis or decreased degradation of serotonin. Therefore, the levels of 5-HIAA, a serotonin metabolite were estimated, in all the regions of rat brain under investigation. An overview of the literature reveals a consensus regarding increased 5-HIAA levels in brain, both in chronic and acute liver failure. Unlike serotonin changes, alterations in 5-HIAA are not uniform. In the present study, the most pronounced increase of 5-HIAA was observed in pons medulla (67%), followed by cortex (52%) and cerebellum (34%). The observed increase in serotonin despite its increased mobilization towards 5-HIAA suggests an increase in the turnover of serotonin in brain during FHF. The increase in serotonin levels could be attributed to its increased synthesis from elevated tryptophan in the tissue. These studies, thus suggest the onset of disturbance in the serotoninergic system, characterized by an increase in the levels of 5-HT and 5-HIAA in TAA –induced acute liver failure.

Kynurenine pathway: Although tryptophan is the precursor for serotonin biosynthesis, it is known that its metabolism in brain is more complex (Guidetti et al., 1995; Stone et al., 1996; Wang and Dunn 1998). A number of studies have demonstrated that increased plasma levels (and therefore brain) of tryptophan not only augmented brain levels of serotonin but also the biosynthesis of the

kynurenines (Stone, 1993). Almost all the metabolites of the kynurenine pathway have been reported in the brain (Freese et al., 1990; Saito et al., 1996). Most of the kynurenines lead to alterations in cellular metabolism and, as a consequence cause damage and cell death (Okuda et al., 1998). The important point is that some metabolites in this pathway are neurotoxic (pro-oxidants) even at low concentrations and some are neuroprotective and therefore, it became necessary to study these metabolites in TAA –induced acute liver failure. The initial reaction in this metabolic pathway is catalyzed by either hepatic tryptophan 2,3 dioxygenase (TDO) or indoleamine 2,3 dioxygenase (IDO), a less specific enzyme with a wider tissue distribution such as brain and kidney (Saito and Heyes 1996). In the present study, it was observed that a significant increase in the activity of IDO only in the pons medulla region while a slight decrease was observed in the cortex and cerebellum. However, Basile et al., (1995) observed no change in the activity of IDO in total brain in TAA treated rats. Kynurenine levels in the brain also followed a similar trend with a significant increase in the pons medulla region but no significant change in cortex and cerebellum. This slight increase in brain kynurenine levels despite 3 fold increase in serum kynurenine levels after TAA treatment could be due to the increase in TDO activity of the liver as observed by Basile et al (1995).

Several other kynurenine metabolites have been shown to exhibit neuroexcitatory, convulsant or toxic properties (Okuno et al., 1996). One such metabolite is 3-hydroxykynurenine (Nakagami et al., 1996), the synthesis of which is catalysed by kynurenine 3-hydroxylase. In the present study, the levels

of 3-hydroxykynurenine increased significantly in different brain regions studied after TAA administration. Increased concentrations of 3-hydroxykynurenine (3-Hk) may lead to brain dysfunction and cause apoptotic neuronal death by the generation of ROS (Okuda et al., 1998). This increase in 3-HK in brain could be due to two reasons: 1) increase in the activity of the enzyme kynurenine 3 - hydroxylase or 2) due to increase in serum and its subsequent transport into brain. This enhanced transport may be either due to altered kinetics of the neutral amino acid carrier, or alterations in the blood -brain barrier permeability as observed in the present study (Chapter 4.5). Tryptophan, 3-hydroxykynurenine and kynurenine have been shown to be actively transported across the blood- brain barrier by the large neutral amino acid carrier, while anthranilic acid has access to the brain by passive diffusion (Schwarcz and Du, 1991). Potentially all these metabolites could enter the CNS from blood and metabolized further via the kynurenine pathway. During et al. (1989) observed an increase in the quinolinic acid concentrations of rat brain when tryptophan was administered peripherally. The same neutral transport carrier which influxes 3-hyroxykynurenine also effluxes glutamine from brain. While effluxing the increased levels of glutamine in the brain during FHF. This would concomitantly lead to an increased influx of 3-hydroxykynurenine. 3-hydroxykynurenine has also increased in other neurodegenerative diseases (Topczewska-Bruns et al., 2001). In addition, oxidative stress may be an important factor causing neuronal death in neurodegenerative diseases. In the present study after TAA administration, observations also revealed an increase in the levels of anthranilic

acid, while the levels of kynurenic acid decreased in all the brain regions under study. Kynurenic acid is an important neuroprotectant formed in this pathway (antagonist of the NMDA receptor). Decreased formation of kynurenic acid could aid in augmenting the action of quinolinic acid a potent lipid peroxidant and NMDA receptor agonist. Anthranilic acid can get rapidly converted to 3-hydroxyanthranilic acid which is again a free radical generator (Okuda et al., 1998); however no physiological importance has been attributed to anthranilic acid. Quinolinic acid also increased in all the regions under investigation. Increase in the levels of quinolinic acid has been reported in galactosamine treated gerbils as a model of acute hepatic injury (Saito et al., 1996). Quinolinic acid is a potent lipid peroxidant and free radical generator causing oxidative stress in most of the neurodegenerative diseases (Stone, 2001a). Recently, it has been shown that quinolinic acid can also impair antioxidant defense mechanism *in vitro* and *in vivo* in young rats (Leipnitz et al., 2005).

Thus, in the present study, alterations in the tryptophan metabolites in both the pathways namely, the serotonin and the kynurenine pathway are observed. The changes in the kynurenine pathway metabolites might be playing a plausible role in generating oxidative stress as some of the metabolites are potent lipid peroxidants and free radical generators. Hence, it would be interesting to analyze the generation of oxidative stress during TAA –induced FHF.

4.3 FHF induced Oxidative stress in different regions of the brain:

The levels of 3-Hydroxykynurenine and quinolinic acid have been found to increase in different regions of brain in response to TAA treatment. As these tryptophan metabolites are potent inducers of oxidative stress and free radical generators, the induction of oxidative stress was studied in different regions of brain during TAA-induced FHF.

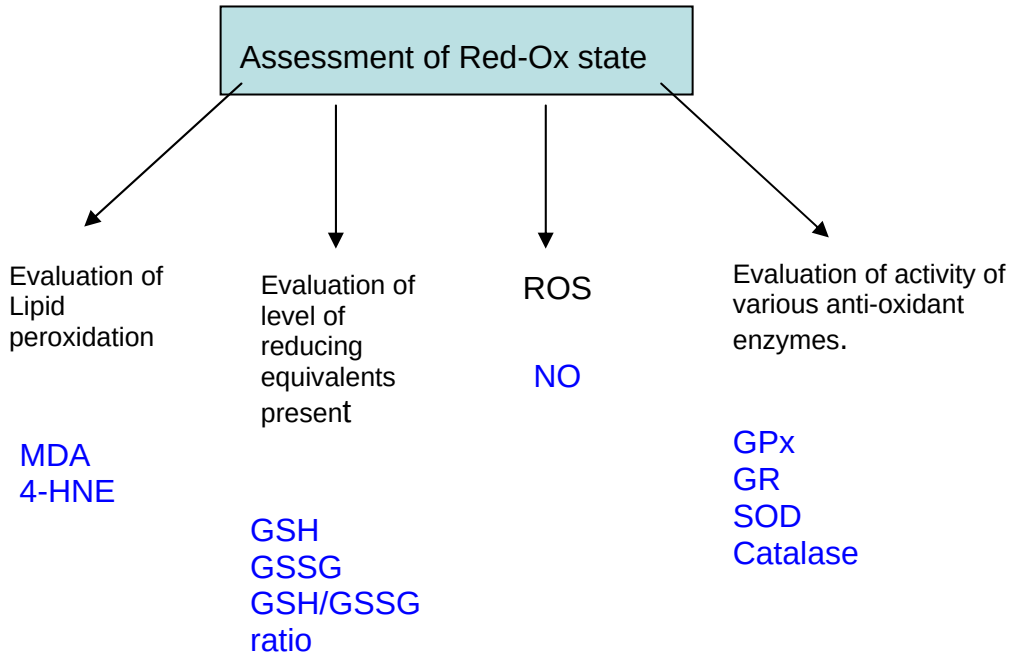
Free radicals are produced during various physiological processes and some have important roles as signaling molecules (Dawson and Synder, 1994). In normal physiological conditions, they are efficiently scavenged by the antioxidants and antioxidant enzymes. Under normal conditions a delicate balance between oxidants and antioxidants will be maintained to prevent any oxidative damage. However, during certain pathological conditions the production of free radicals may override the cellular antioxidant defenses leading to oxidative stress. Oxidative stress is an evolving concept in the pathophysiology of many neurodegenerative disorders such as Alzheimer's disease, Amylotrophic lateral sclerosis and Parkinson's disorders and during ischaemia-reperfusion injury, and exposure to chronic stress exposure (Smythies, 1999).

Brain is particularly susceptible to oxidative damage as it is enriched with poly unsaturated fatty acids, which are liable to peroxidation from oxygen free radicals. Also the brain has lower level of repair mechanisms and consumes very high oxygen (Halliwell and Gutteridge 1985; Reznick and Packer, 1993; Halliwell, 1992). Additionally some cerebral regions are enriched with non-heme

iron, which can be catalytically involved in the production and propagation of oxygen free radicals (Hill and Switzler, 1984) and thus increasing the risk for oxidative damage.

Recently, oxidative stress has also been implicated in mitochondrial damage and pathology of hepatic encephalopathy. Oxidative stress has been earlier demonstrated in cortex of hyperammonemic and hepato-encephalic animals. Most of the earlier work in this direction has been on whole brain homogenates, or cortex and particular subcellular fractions isolated from cerebral cortex. Brain is structurally, functionally and metabolically heterogeneous and different regions in brain demonstrate different levels of susceptibility to oxidative and other stress conditions mostly based on the metabolic constraints and functions. Hence, in the current study, an attempt is made to analyse the differences in the extent of oxidative stress as assessed by levels of lipid peroxidation, GSH, GSSG, NO and changes in activity of antioxidant enzymes, during HE in the three major cerebral regions namely cortex, cerebellum and pons medulla. Lipid peroxidation is a self propagating chain reaction i.e., the initial oxidation of only a few lipid molecules can lead to significant tissue damage and disease. Among different markers of oxidative stress, malondialdehyde (MDA) and the antioxidant enzymes are considered to be the most important. MDA is a three carbon compound formed from peroxidized polyunsaturated fatty acids, mainly arachidonic acid. Superoxide dismutase protects against oxygen free radicals by catalyzing the removal of superoxide anion radical, while catalase is primarily responsible for the detoxification of hydrogen peroxide.

Glutathione is required in various reactions involving the reduction of peroxides such as hydrogen peroxide and other organic peroxidases and protects cell membranes from lipid peroxidation while glutathione reductase is an important enzyme for maintaining the intracellular concentration of reduced glutathione. As indicated in the earlier chapters,TAA-induced HE has resulted in alterations in various bio-chemical parameters in the brain leading to impaired BBB and tissue damage. These results suggest possible onset of oxidative stress in the brain during TAA-induced HE. In order to test this, further studies were undertaken to assess the onset of oxidative stress in different regions of brain of animals treated with TAA. The oxidative stress in TAA-induced FHF is assessed by measuring the parameters as given below.



4.3.1 Results:

A significant increase in the levels of MDA (the marker for lipid peroxidation) was observed in all the regions of the brain in response to TAA treatment, the maximum increase being in cortex (41%), followed by cerebellum (37%) and pons medulla (40.1%) (Fig 4.3.1). ROS levels (Fig 4.3.3) measured by DCF also showed similar trend; 55% in cortex, 45% in cerebellum and 42% in pons medulla. Immunohistochemistry studies using 4-HNE antibodies revealed 4-HNE modified proteins in all the regions of brain in TAA treated rats (Fig4.3.2). NO levels measured by the formation of nitric oxide (Fig 4.3.5) also showed a significant increase in pons medulla (135%), cortex (63%) and cerebellum (42%).

GSH and GSSG levels both increased (Fig 4.3.4A &4.3.4B) but the ratio of GSH/GSSG (Fig 4.3.4C) showed a significant decrease in all the brain regions. GSH/GSSG levels are important parameters indicating the redox state of the cell and the decrease in GSH/GSSG ratio clearly indicates a decrease in reducing equivalents in the cell.

Enzyme activities

Glutathione peroxidase (Fig 4.3.6 A) showed a 11%, 13.5% and 29% decrease in activity in cortex, cerebellum and pons medulla respectively. The activity of glutathione reductase (Fig 4.3.6B) decreased by 19.0% in cortex, 15%

in cerebellum and 31% in pons medulla. Activity of catalase (Fig 4.3.6 D) decreased by 26% in cortex, 16% in cerebellum and 19% in pons medulla, while SOD activity (Fig 4.3.6 C) increased by 42% in cortex, 58% in cerebellum and 89% in pons medulla.

Lipid peroxidation level:

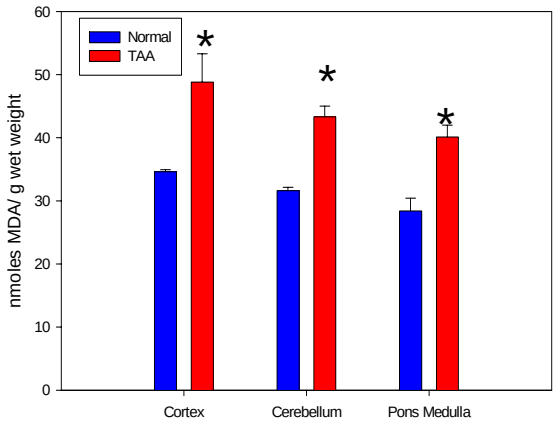


Fig: 4.3.1 MDA levels in cortex, cerebellum and pons medulla in control and thioacetamide treated rats. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05. * Significant over control.

Immunohistochemical detection of 4-hydroxynonenal-modified proteins

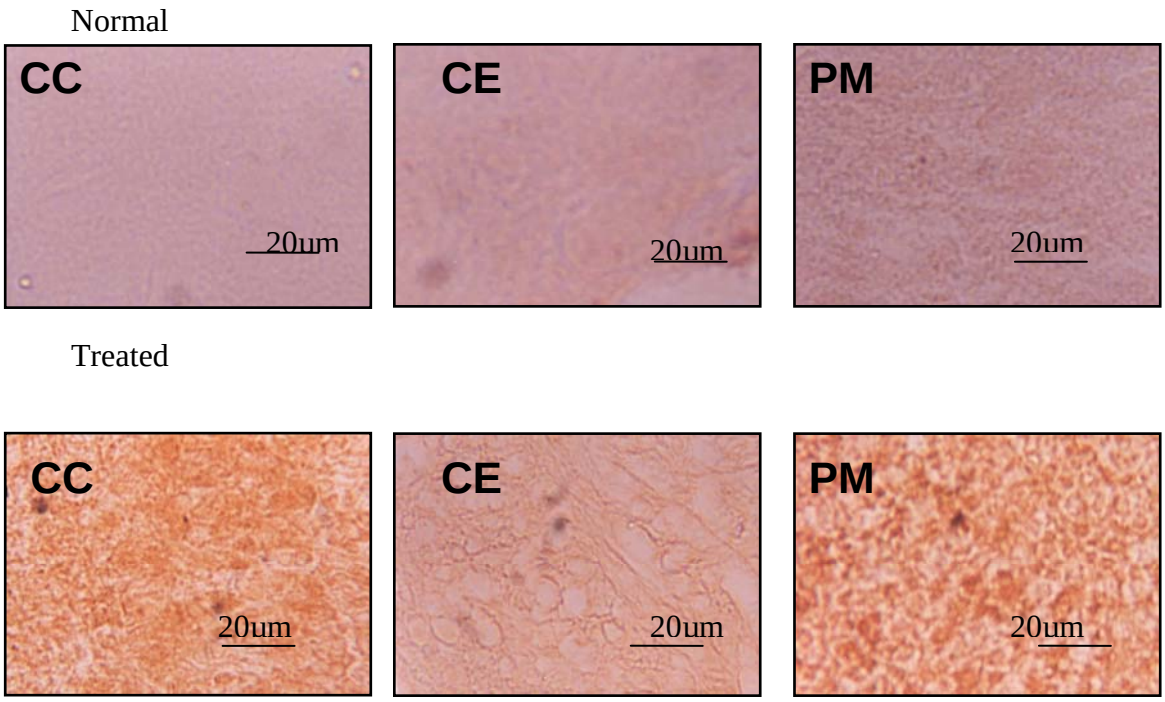


Fig: 4.3.2 Effect of TAA-induced FHF on accumulation of 4-hydroxynonenal in different regions of brain. Immunohistochemistry using antibodies against 4-hydroxynonenal adducts is described under materials and methods. Representative photomicrographs depicting immunohistochemistry of 4-hydroxynoneal modified (brown) proteins in the brain cross sections.

ROS Levels

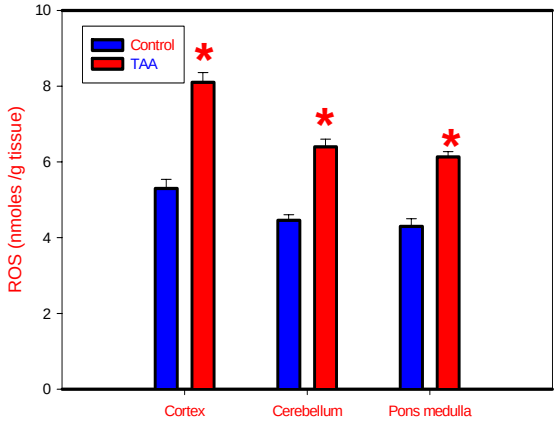


Fig: 4.3.3 Levels of ROS in cortex, cerebellum and pons medulla in control and thioacetamide treated rats. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.
* Significant over control.

Glutathione levels:

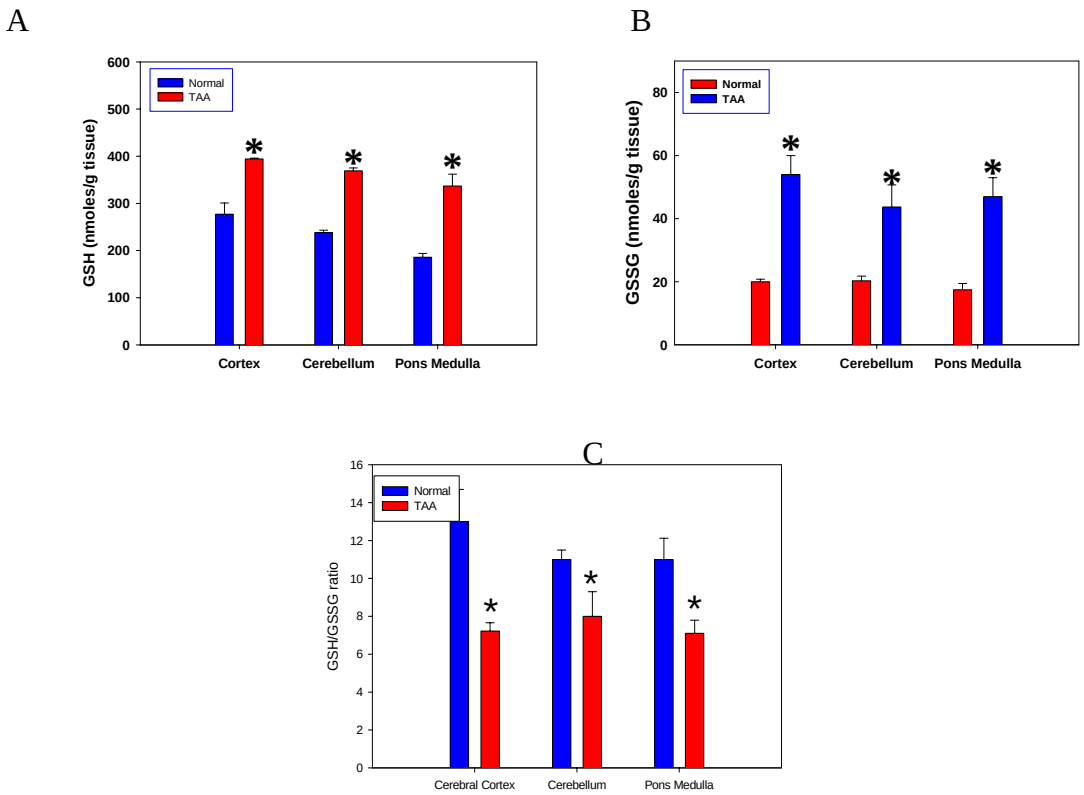


Fig:4.3.4 GSH (A) and GSSG (B) levels both increased but the ratio of GSH/GSSG (C) showed a significant decrease in all the brain regions. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Nitric oxide levels:

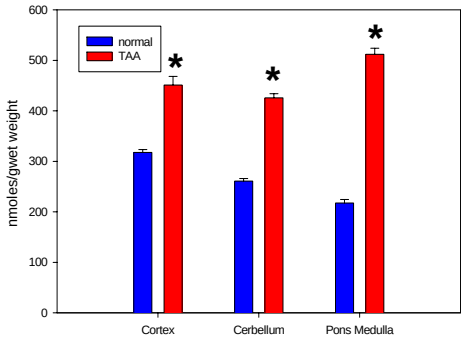
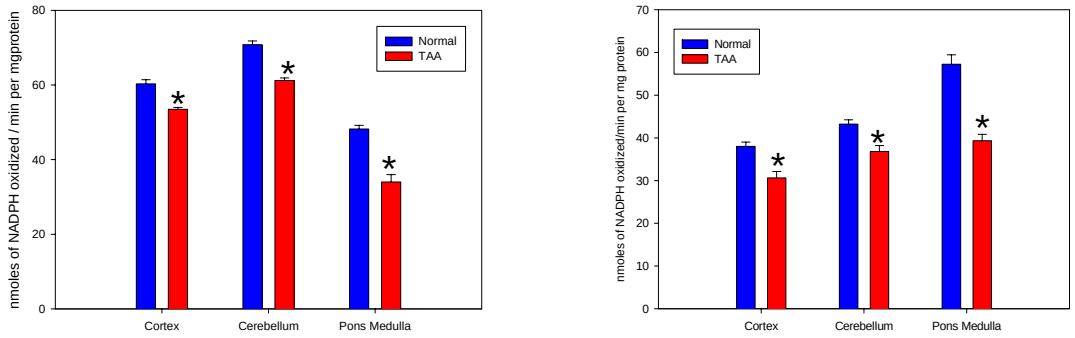


Fig: 4.3.5 Total nitric oxide levels in cortex, cerebellum and pons medulla increased in all the regions of brain under investigation in thioacetamide treated rats. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Antioxidant enzyme activity levels:

A) Glutathione peroxidase **B) Glutathione reductase**



C) Superoxide Dismutase **D) Catalase**

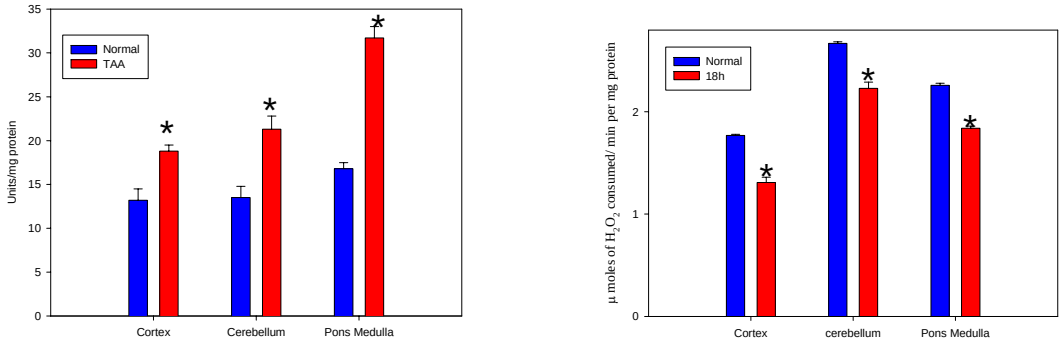


Fig:4.3.6 Alterations in the activity of antioxidant enzymes of A) Glutathione peroxidase B) Glutathione reductase C) Superoxide dismutase and D) Catalase in different regions of brain during TAA induced FHF. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at $P< 0.05$.* Significant over control.

4.3.2 Discussion:

The present study highlights the effects of oxidative stress observed during TAA induced acute liver failure. The cellular damage induced by free radicals was estimated by monitoring the MDA levels which are the markers for lipid peroxidation. Peroxidation of unsaturated fatty acids in membrane phospholipids is one of the multiple cytotoxic effects of oxidative stress and generation of lipid adducts is significant in a cell because a single initiating event triggers a chain reaction amplifying the initial stimulus (Halliwell and Gutteridge, 1997). In the present study, there was increase in the level of lipid peroxidation as evidenced by increase in the levels of MDA in all the regions of brain. Also there was increase in the lipid peroxidation adducts such as HNE in all the regions of brain. The increase in the reduced form of glutathione in the tissue homogenates in TAA treated rats may be due to increased activity of the gamma glutamyl-cysteine synthatase. Similar results were obtained by Murthy et al., (2000) in astrocyte cultures exposed to hyperammonemia. Normally 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 reduced GSH content, the oxidized form of glutathione GSSG was also enhanced in the present study. Although an increase in both forms was observed, the magnitude of increase of the oxidized glutathione was much higher than the increase in GSH. This is further evident from the ratio of GSH/GSSG. The decreased ratio indicates that the tissue is being pushed to a state where the

oxidized form of glutathione (GSSG) gets accumulated. This decrease in the ratio of GSH/GSSG ratio along with the increased lipid peroxidation suggests the possible induction of oxidative stress in TAA treated rats.

Another important free radical, Nitric oxide (NO), the product of inducible nitric oxide synthase, is known to ameliorate or potentiate the effects of ROS (Kikugawa et al., 2005). Increased nitric oxide levels could be due to the activation of NMDA receptors, either by quinolinic acid or ammonia. Activation of NMDA receptors leads to increased intracellular calcium levels, which binds to calmodulin and activates different enzymes including nitric oxide synthase. NO is also known to inhibit catalase activity, leading to altered detoxification of free radicals (Wang et al., 1998). Further, it can also react with superoxide anions to form peroxynitrite, which in turn react with sulphydryl residues in cell membranes leading to peroxidation of lipids. This is observed in the current study as evident from the increased ROS levels measured by Dichlorofluoroscein and MDA levels, the markers for lipid peroxidation. Thus, the present study suggests that the induction of nitric oxide may potentiate the effects of ROS rather than ameliorating the effects of TAA. Increase in ROS levels in brain may be due to impaired antioxidant defenses apart from other known causes like increase in the tryptophan metabolites specially 3-hydroxykynurenine and quinolinic acid. Quinolinic acid is also known to decrease the antioxidant defense mechanism in young rats (Leipnitz et al., 2005). Further, hyperammonemic states prevailing in acute hepatic failure conditions activates the NMDA receptors leading to increased calcium influx, which in turn activates various pathways involved in

free radical generation in brain namely iNOS and uncoupling of mitochondrial electron transport chain (Kosenko et al., 1998; 2003).

Decreased antioxidant defenses:

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. Significant perturbations in the antioxidant enzyme activities in different regions of brain were evident in animals treated with TAA.

Glutathione reductase is a flavoprotein that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) and maintains the constant levels of reduced glutathione levels. The decrease in the glutathione reductase activity indicates a decrease in the conversion of GSSG to GSH suggesting impaired redox cycling. However, the increase in GSH levels could be due to the increase in the activity of gamma glutamyl-cysteine synthatase (Lu et al., 1999). The levels of GSSG have increased despite a decrease observed in glutathione peroxidase activity. The observed increase in the SOD activity in response to TAA administration may be an attempt to detoxify the increased ROS and thus protect the tissue from of $O^{2-\cdot}$ radicals. Superoxide dismutase is considered to be the first line of defense for protecting the cell from deleterious effects of oxyradicals. (Fridovich,1986). Increased expression of SOD results in the enhanced production of hydrogen peroxide that could result in lipid peroxidation especially when it is not reduced

by peroxidases (Ceballos-Picot et al., 1991). The decrease in the catalase and glutathione peroxidase activities in the brain during TAA-induced FHF suggests impaired antioxidant defenses. Hydrogen peroxide as a metabolite is more toxic than oxygen radicals and H_2O_2 in the presence of transition metals like iron leads to the generation of highly toxic hydroxyl ($^{\cdot}OH$) radicals which are known to induce lipid peroxidation. Decreased enzymatic activities of glutathione peroxidase, glutathione reductase and catalase and an increase in the activity of SOD observed in the present study might invariably lead to elevated levels of H_2O_2. Similar observations have been made by Reddy et al. (2004) in mitochondria of cortex and Dogru- Abbasoglu et al., (2001) in the liver of TAA induced FHF. Although an increase in SOD activity has a protective role, its increase with a simultaneous reduction in the activity of glutathione peroxidase is lethal to the tissue due to the accumulation of more hydroperoxides (Avraham et al., 1998; Devi et al., 1996). Though all the regions of brain under study are affected, cortex and pons medulla show more pronounced damage compared to cerebellum. This is in accordance to general observations that cortex is more susceptible to stress than cerebellum (Mandavilli and Rao, 1996).

Thus, from the present study, it is clear that TAA-induced FHF results in the oxidative stress in different regions of brain as a result of increased generation of ROS coupled with impaired antioxidant defenses. This induced oxidative stress might result in Tissue damage. Hence, it would be interesting to probe further on the effects of TAA-induced FHF on brain histopathology.

4.4 Histopathology:

The foregoing studies clearly indicated increased oxidative stress in different regions of brain during TAA treatment. Oxidative stress may lead to ultrastructural changes in cellular and subcellular compartments which could affect cerebral metabolism, thus contributing to the pathophysiology of HE.

Brain metabolism depends almost completely on the metabolism of systemically derived substrates. About 75% of hepatic glucose production is used in the brain and about 90% of cerebral glucose metabolism takes place by the Krebs cycle and the gamma-amino butyric acid shunt. Uptake of amino acid and ammonia taken from blood vary in different regions of the brain. Morphological changes in brain tissue do not always occur in patients with encephalopathies. However, in some types of hepatic encephalopathies tissue hypoxia and tissue edema and possibly tissue necrosis may be found. At cellular level morphological changes of astrocytes may be seen.

There is evidence from clinical and experimental research that vascular factors, infections, endotoxins and disturbances of the blood- brain barrier with changes in the amino acid and neurotransmitter profile may play a distinctive role in the pathophysiology of encephalopathies. However, definite causes of encephalopathy are not clear till date.

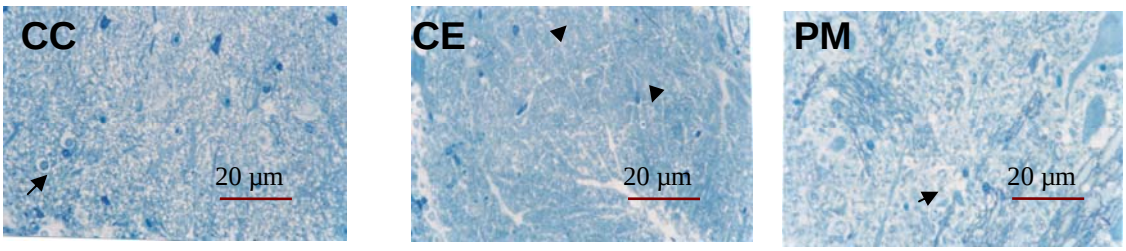
Ammonia in brain is detoxified to glutamine, in the astrocytes (Norenberg and Martinez-Hernandez, 1979). The glutamine so formed accumulates in astrocytes and may exert an osmotic effect leading to cell swelling. Brain edema leading to intracranial hypertension is a cause of death in acute liver failure

(ALF). The pathogenesis of this unique complication has been investigated in man, experimental animals and in isolated cell systems, yet it is unclear. Earlier studies evaluating brain function have shown the induction of oxidative stress, suggesting its possible effect on its structure. Hence, in the present study investigations are carried out for the presence of cellular and morphological changes in different regions of brain during TAA-induced FHF using light microscopy and TEM.

4.4.1 Results:

The light microscopic studies indicated a marked swelling in the astrocytes in all regions of brain (Fig 4.4.1). In the light of changes observed under light microscope, transmission electron microscopic studies were performed on different regions of brain in order to study changes in any organellar ultra structure (4.4.2). In the rats treated with TAA, clear ultrastructural changes were observed compared to control rats.

Normal



TAA treated

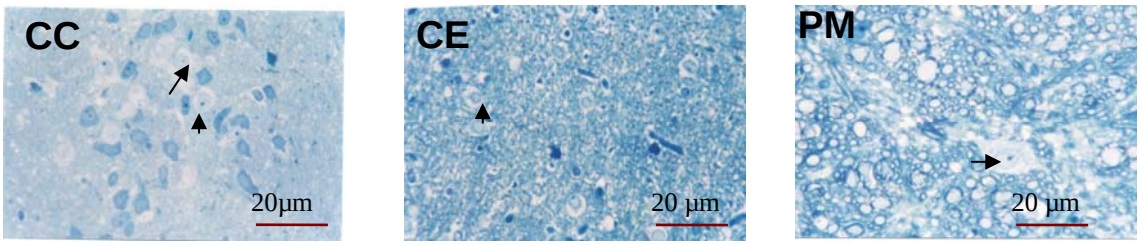
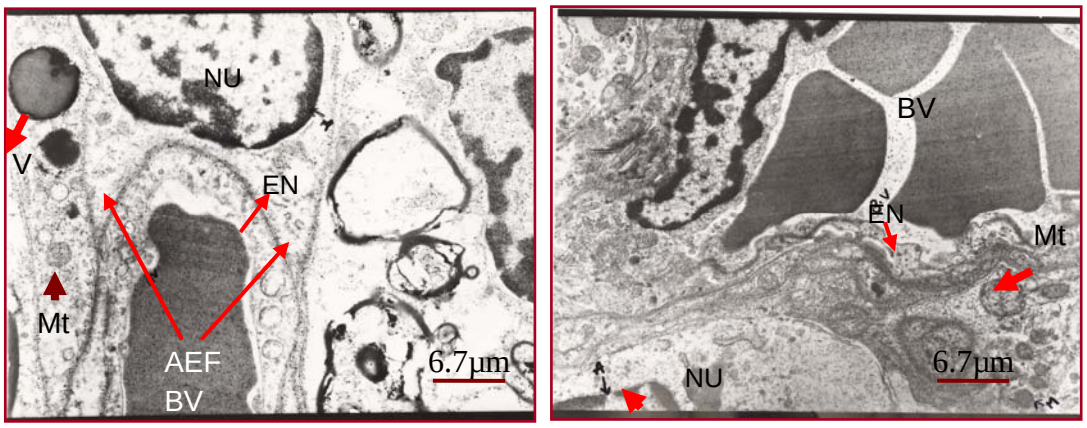


Fig:4.4.1 Pictures of cc, ce and pm of normal and TAA treated rats. There is clear swelling of astrocytes in different regions of brain of TAA treated rats.

Transmission electron microscopic images:

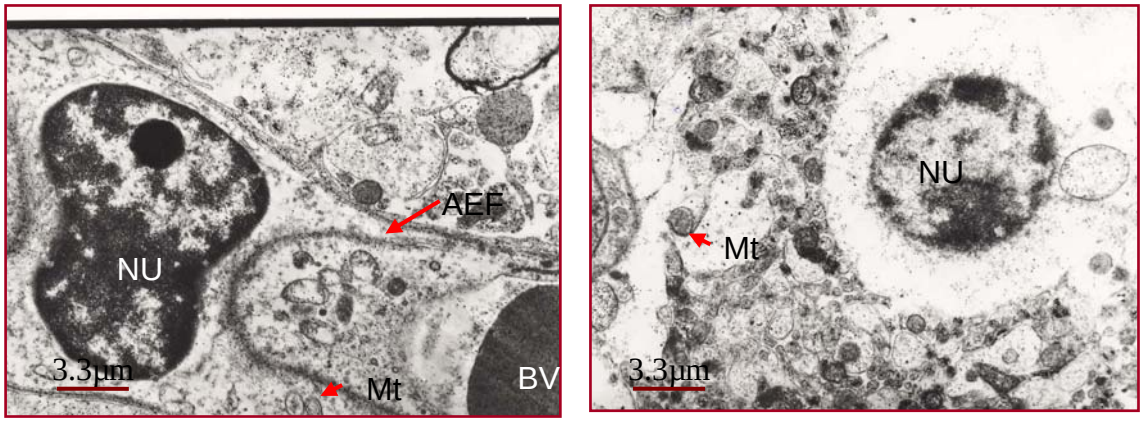
Astrocytes in Cortex



Normal TAA

BV – Blood vessels, AEF– Astrocyte end feet, Mt – Mitochondria
EN – Endothelium, NU – Nucleus, V -- Vesicle

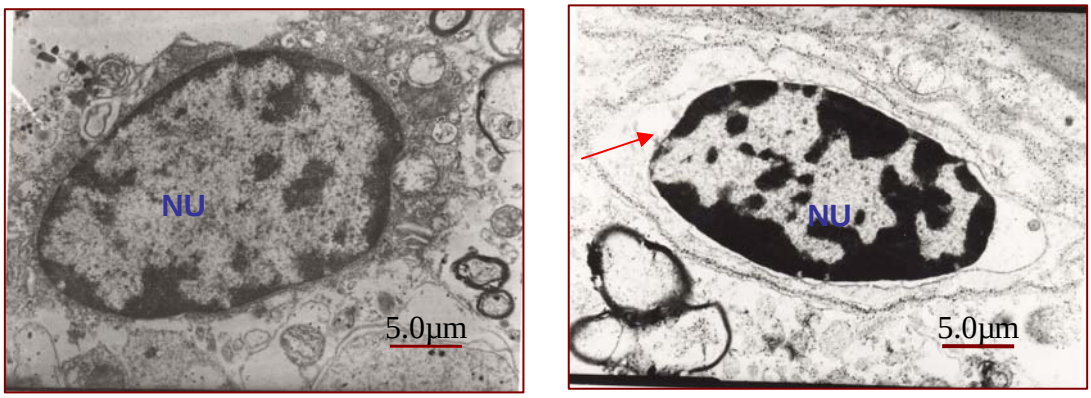
Astrocytes in Cerebellum



Normal **TAA**

BV – Blood vessels, AEF– Astrocyte end feet, Mt – Mitochondria
EN – Endothelium, NU – Nucleus, V -- Vesicle

Astrocytes in Pons Medulla



Normal **TAA**

Fig: 4.4.2 Astrocytes observed under electron microscope revealed marked cytoplasmic and mitochondrial swelling in all the brain regions of TAA treated animals under study confirming the light microscopic observations.

4.4.2 Discussion:

Both transmission electron microscopic and light microscopic studies showed significant and marked cytoplasmic swelling in astrocytes in the 3 regions of brain. These results are in agreement with earlier reports in cortex involving human HE patients (Kato et al., 1992) and other experimental models (Traber et al., 1987). The reduction in the extracellular space following astrocyte swelling may elevate extracellular ionic concentrations, which could affect neuronal excitability (Smith, 1992). Astrocyte swelling could also compress capillaries, contributing to a reduction in cerebral blood flow (Garcia et al., 1980).

Cerebral edema is believed to be the chief pathological feature leading to coma and death during increased intracranial pressure (Blei and Larsen, 1999; Blei, 2005; Master et al., 1999, Butterworth, 1992). Vasodilation and other circulating factors have been implicated in the etiology of cerebral edema in hyperammonemic states and HE (Bender and Norenberg 1998; Cordoba et al., 1998; Haussinger et al., 2000). In addition, changes in cytoplasmic volume are known to have direct impacts on cellular metabolism. The enhanced ratio of cytoplasm to nuclear volume, as observed in the present study, could affect transcription and translation of several factors. Clear cytoplasmic swelling, along with mitochondrial swelling and nuclear condensation were observed in different regions of brain. Mitochondrial swelling indicates probable damage to the mitochondrial integrity leading to disruption of mitochondrial function. This might further result in alterations in the mitochondrial electron transport chain, leading

to enhanced production/leakage of free radicals and thus producing oxidative stress.

From the above studies it is evident that TAA induces cytoplasmic and mitochondrial swelling with simultaneous nuclear condensation in different regions of brain of TAA treated rats, which may be due to impaired blood-brain barrier leading to cerebral oedema. Hence, it would be interesting to study the effect of TAA on blood-brain barrier.

4.5 Blood-Brain Barrier

Histopathological studies indicated a marked swelling in astrocytes, a chief component of blood-brain barrier. The increased oxidative stress observed in the present study, could also affect blood-brain barrier.

The blood-brain barrier (BBB) is the specialized system of capillary endothelial cells (Fig 4.5.1) that protects the brain from harmful substances in the blood stream, while supplying the brain with the required nutrients for proper function. Unlike peripheral capillaries that allow relatively free exchange of substances across / between cells, the BBB strictly limits transport into the brain through both physical (tight junctions) and metabolic (specific transporters) barriers. In addition to providing protection from systemic toxins, BBB is also the primary concern in administration of therapeutic agents specifically targeted to the brain tissue as it effectively blocks accessibility to the cerebral tissue. Thus the BBB is often the rate-limiting factor in determining permeation of therapeutic drugs into the brain. BBB breakdown is theorized to be a key component in central nervous system (CNS) associated pathologies like ischaemia, Alzheimer's etc. The periendothelial accessory structures of the BBB include pericytes, astrocytes, and a basal membrane. The endothelial cell of the BBB is distributed along the length of the vessel and completely encircles the lumen.

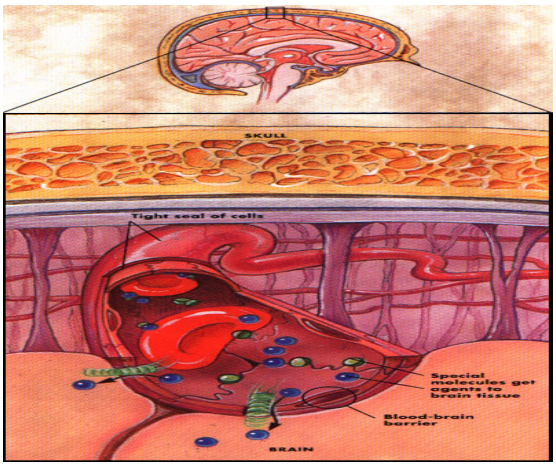


Fig: 4.5.1. Figure depicting the anatomical structure of the Blood -brain barrier

Astrocytes envelope greater than 99% of the BBB endothelium and exchange occurs via specialized astrocytic structures called end feet. Intercellular adhesion between astrocytes in the blood-brain barrier has been observed in the form of gap junctions and adhering junctions (Brightman and Reese, 1969). There is considerable evidence, *in vitro* and *in vivo*; to indicate that astrocyte interaction with the cerebral endothelium helps determine BBB function, morphology (i.e. tightness) and protein expression (Beck et al., 1984; Arthur et al., 1987; Cancilla et al., 1983). Astrocytes serve as scaffolds, guiding neurons to their proper place during development and direct vessels of the BBB. The association of astrocytes to the cerebral microvasculature is underlined by the association of neurons to astrocytes. Transfer of systemic substrates and metabolites from blood to neurons is believed to occur via the astrocytes.

Impaired blood- brain barrier, leading to enhanced permeability to fluid or solutes has been implicated in several diseases like hypertension, edema, inflammation, ischemia, and reperfusion damages (Banks and Kastin 1996). Dixit and Chang (1990) have reported blood-brain barrier breakdown in galactosamine

induced acute liver failure. As indicated in the earlier chapter, TAA treatment to rats resulted in increased exposure of brain to ammonia, which in turn altered amino acid composition (Chapter 4.1). These biochemical changes followed closely with ultrastructural changes leading to edema, cytosolic, and mitochondrial swelling and nuclear condensation. (Chapter4.4). These biochemical ultrastructural changes observed in the brain in response to TAA treatment , suggests possible impairment in BBB. Hence further studies were undertaken to study the impact of TAA treatment on BBB.

4.5.1 Results:

Evans blue studies:

Evans blue studies were carried to determine the integrity of the blood-brain barrier after TAA administration. Administration of Evans blue to normal animals showed no significant coloration of the brain tissue (Fig 4.5.2A) while TAA treated animal (Fig 4.5.2B) showed a distinctive blue coloration in all regions of the brain.

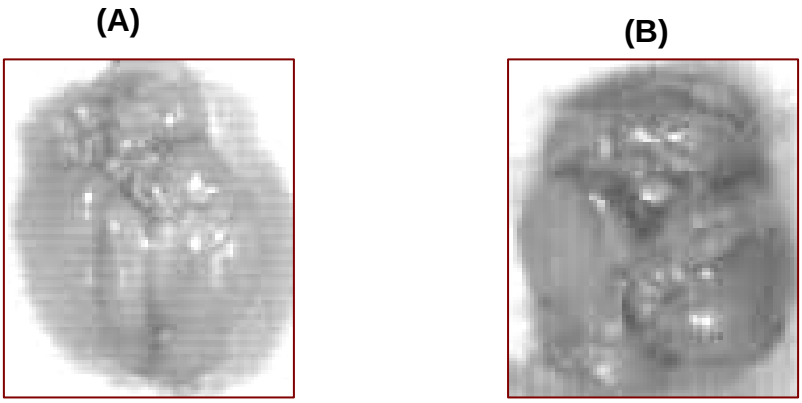
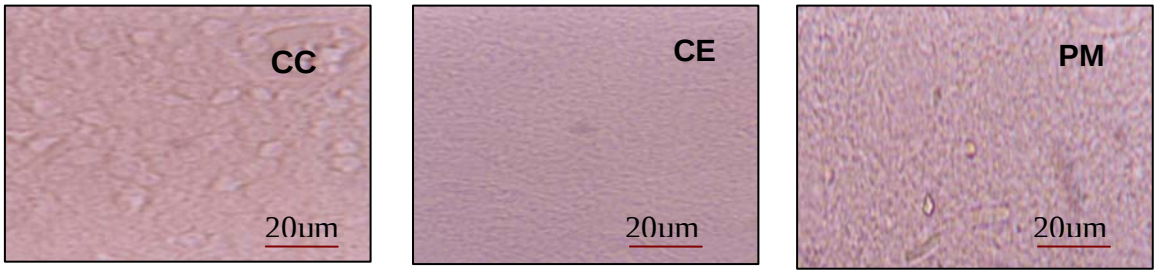


Fig: 4.5.2 Evans blue experiments showing the structural integrity of the blood-brain barrier in normal (A) and TAA treated (B) rats. A 2% solution of Evans blue was given at a dose of 3 ml/ kg (60 mg/ kg) i.p to both normal and TAA treated rats after 12h administration of second dose of TAA.

Light microscopic studies

Normal



TAA Treated

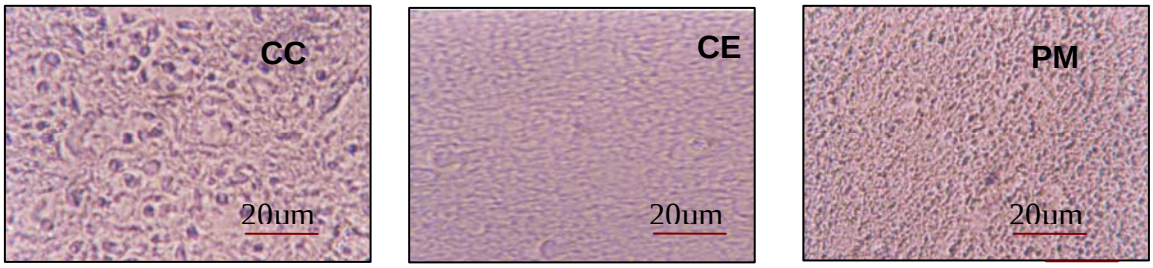


Fig: 4.5.3 Light microscopic pictures of the brain histological sections showing Evans blue in cells of different regions of brain in both normal (top panel) and TAA treated (bottom panel) rats. A 2% solution of Evans blue was given at a dose of 3 ml/ kg (60 mg/ kg) i.p to both normal and TAA treated rats after 12h administration of second dose of TAA. Tissues were fixed and processed by the same method as mentioned in the materials and methods under histopathology but with no H& E staining.

Spectrophotometric Method

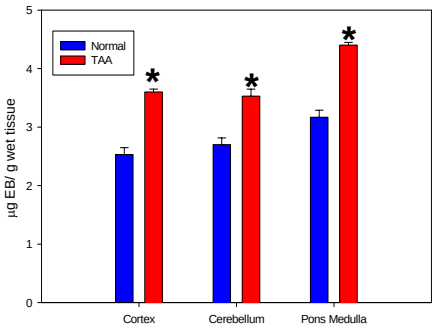


Fig: 4.5.4 Spectrophotometric estimation of Evans blue revealed a significant increase in all the regions of brain in TAA treated rats. Each brain region was weighed and the samples were then homogenized with 2.5 ml phosphate-buffered saline (PBS) and mixed with 2.5 mL 60% trichloroacetic acid to precipitate protein. The samples were centrifuged for 30 min at 1000*g* and the supernatants were measured at 610 nm for absorbance of EB by using a spectrophotometer. EB is expressed as micrograms per milligram of brain tissue against a standard curve.

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control

Transmission electron microscopy:

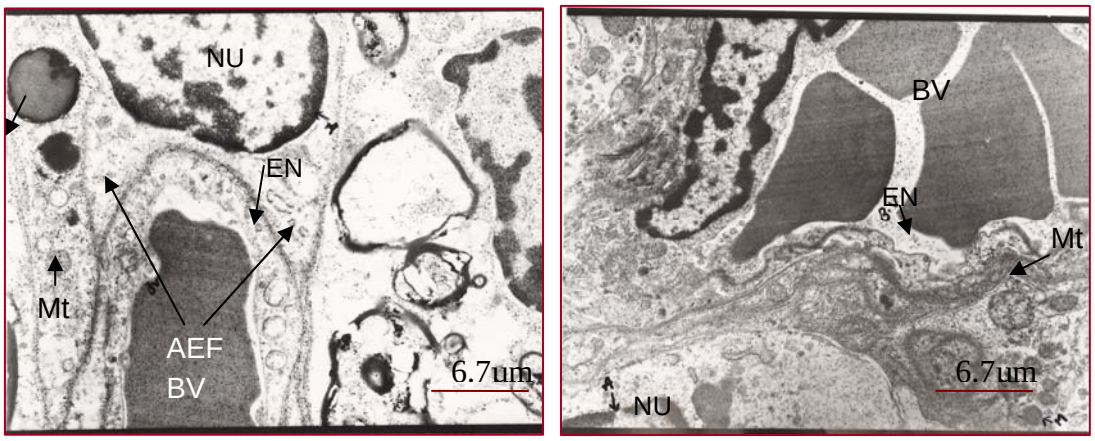


Fig: 4.5.5 Transmission electron microscopic images also revealed considerable changes in the blood- brain barrier morphology in cortex of TAA treated rats. The endothelial lining of the blood vessels in the TAA treated rat is altered. The lining was convoluted and flaccid when compared to intact turgid structure observed in normal animals. Also seen are the changes in the astrocytes lining the blood vessels. AEF- astrocytic endfeet, BV- Blood vessel, Mt- Mitochondria, EN-endothelial lining, Nu-Nucleus.

4.5.2 Discussion:

Histopathological studies (Figs 4.5.2B & 4.5.3B) with Evans blue indicate a break down of blood-brain barrier during TAA induced FHF as evidenced by the distinct blue coloration of the brain tissue due to increased permeability of the dye in the cerebral tissue. This was further confirmed by quantifying the Evans blue in the tissues by spectrophotometric method (Fig 4.5.4) and transmission electron microscopic studies (Fig 4.5.5). Evans blue does not

normally cross an intact blood-brain barrier unless alteration occurs in the BBB permeability. Evans blue when bound with serum albumin forms a complex which in normal conditions can not permeate into the brain, and therefore its permeation into the brain indicates alterations in the blood-brain barrier. Transmission electron microscopic images in cortex of TAA treated rats showed clear disruptions in the lining of blood vessels (Fig 4.5.5). These results coincide with that of Dixit and Chang (1990) where the authors have shown the BBB break down with trypan blue dye during the progression of galactosamine induced acute liver failure. Of late, the BBB is being studied with greater attention for several reasons, mainly to understand its architecture and physiology (Pardridge 1999; Stewart 2000) and also for therapeutic applications. However, more attention is focused on the BBB under pathological conditions particularly on its role in vasogenic brain edema, which together with cytotoxic edema (cellular swelling) is the most frequent cause of life threatening intracranial hypertension. Observations from the present study revealed marked increase in astroglial and mitochondrial swelling in different regions of brain (Fig 4.4.2) after TAA treatment. Extensive cytoplasmic swelling of astroglial cells resulting in tissue necrosis might be the reason for the blood brain barrier break-down. Similar observations have been reported in the cytotoxic brain edema resulting out of variety of factors (Lee and Bakay, 1967). This impaired blood-brain barrier in rats treated with TAA might be responsible for the observed alterations in the brain amino acids, swelling of cytoplasm, mitochondria and brain edema.

Thus, the present study confirms the increased permeability of BBB in TAA induced acute liver failure. However, the increase in permeability here may be mediated either via damage to vascular endothelial cells (vasogenic) or astrocyte swelling through increased intracellular osmolarity (cytotoxic) or both. Presence of increased astrocyte swelling argues for the presence of cytotoxic factors more, rather than vasogenic factors. However, vasogenic edema cannot be ruled out altogether. In the current study, it is observed that pons medulla and cortex showed more permeability than cerebellum as evidenced by the extravasion of Evans blue dye.

Chapter 3

Protection with C-Phycocyanin

5.0 Protective effect of C-Phycocyanin

Liver has remarkable ability to metabolize and aid in the detoxification of xenobiotics. Nevertheless, it is susceptible to damage from a number of drugs and toxins. Liver injury following the intake of such agents may be sub clinical and reversible, but may also be fatal at times because of FHF. Rodent models of toxin-induced hepatotoxicity are used to elucidate the biochemical processes involved in many forms of liver disease and to evaluate the therapeutic potential of candidate hepatoprotectants. In the present study FHF was induced in rats by the administration of TAA. This has resulted in the damage of not only liver, but also in different parts of brain, mainly by the oxidative stress leading to alterations in blood-brain barrier and ultrastructural changes. Since the damage is inflicted by TAA in liver and brain by the oxidative stress, it is quite likely that dietary supplementation of antioxidants might offer protection. Hence, in the present study the protective effect of C-Phycocyanin (C-PC), a natural antioxidant, was evaluated on TAA induced tissue damage.

C-Phycocyanin, a copper containing protein, is known for its various medicinal as well as pharmacological properties (Farooq et al., 2004). It is a water soluble non-toxic fluorescent protein pigment isolated from *Spirulina platensis*, a blue green alga. C-PC contains a tetrapyrrole chromatophore known as phycocyanobilin, which is covalently attached to the apoprotein. Some of the earlier studies have suggested that phycocyanobilin plays the key role in some of the biological properties exhibited by phycocyanin (Lissi et al., 2000). C-PC is also known for its strong antioxidant, anti-inflammatory, radical scavenging

properties as well as exhibiting neuro, hepato-protective and anti tumour effects (Romay et al., 1998, 2000; Vadiraja et al., 1998; Vadiraja and Madyastha, 2000; Rimbau et al., 1999; Li et al., 2005). Earlier studies from our laboratory revealed that C-PC is a selective inhibitor of COX-2 (Reddy et al., 2000) and induces apoptosis in mouse macrophage cell line RAW 264.7 (Reddy et al., 2003) in rat histiocytoma cell line, AK5 (Pardhasaradhi et al., 2003) and chronic myeloid leukemia cell line, K-562 (Subhashini et al., 2004). Although a wide variety of antioxidants exist, the purpose of selecting C-PC for the present study is because of its ability to cross the blood brain barrier (Romay et al., 1998) and its hepato-protective effects.

5.1 Results:

Effect of C-PC on the survival rate of TAA treated rats.

All the rats that received TAA and C-PC survived and were alive up to 36hrs following the second administration of TAA whereas the group that received only TAA exhibited a 20 % survival rate up to the same period (FIG 5.1). To determine the long- term survival of rats with TAA-induced FHF, the surviving rats were followed up to 10 days after the induction of FHF, and 70% of the C-PC rats were alive during the follow-up period.

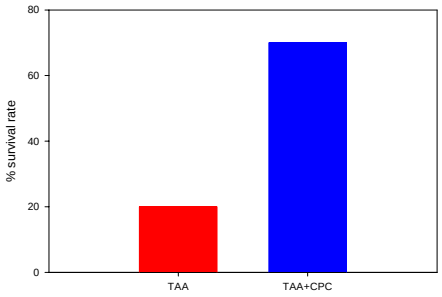


Fig: 5.1 Percent survival of rats treated with TAA alone or in combination with C-Phycocyanin. N= (10) observed for 10days.

Effect of TAA and C-PC on liver enzymes and ammonia and serum albumin, prothrombin time and ammonia

Thioacetamide administration (TAA) resulted in a marked decrease in the activities of liver aminotransferases and increased ammonia levels, compared to untreated rats (Table 5.1). The liver enzymes AAT and AST improved by 77% and 80% respectively while liver ammonia levels improved significantly by 95% in rats treated with TAA and C-PC. Serum AAT and AST activity also improved by 66% and 77% respectively. Serum (95%) and brain ammonia (71%) levels also improved significantly in rats treated with TAA and C-PC. The serum albumin levels increased significantly along with decrease in prothrombin time in rats treated with TAA and C-PC.

Table: 5.1. Effect of C-PC on tissue and serum biochemical parameters in rats treated with C-PC.

Tissue/Serum	Biochemical parameters	Control	TAA (18h)	TAA + C-PC (18h)
Liver	AAT (µmol/g wet weight/h)	1540± 31	479± 25 *	1290± 21*†
	AST (µmol/g wet weight/h)	2109± 21	620± 29 *	1815± 20*†
	Ammonia (µmol/ g wet weight)	0.51±0.10	2.31± 0.12 *	0.59± 0.05†
Serum	AAT (µmol/ml)	4.19±0.38	27.9±0.28 *	12.3±0.25*†
	AST (µmol/ml)	7.09± 0.29	16.19±0.22 *	9.22±0.28*†
	Ammonia (µmol/ ml)	0.161±0.08	0.98±0.05 *	0.20±0.08 †
	Prothrombin time (sec)	15.1 ± 0.7	1080±28 *	380±32 * †
	Albumin mg%	3.46± 0.026	2.77± 0.040 *	3.13± .088 *†
Brain	Ammonia (µmol/ g wet weight)	0.310± .0400	0.566 ± .0470*	0.385 ±0.011 †

* Significant over control † Significant over TAA treated values

Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Liver histopathology:

Significant decrease in necrosis was observed in rats treated with TAA+C-PC.

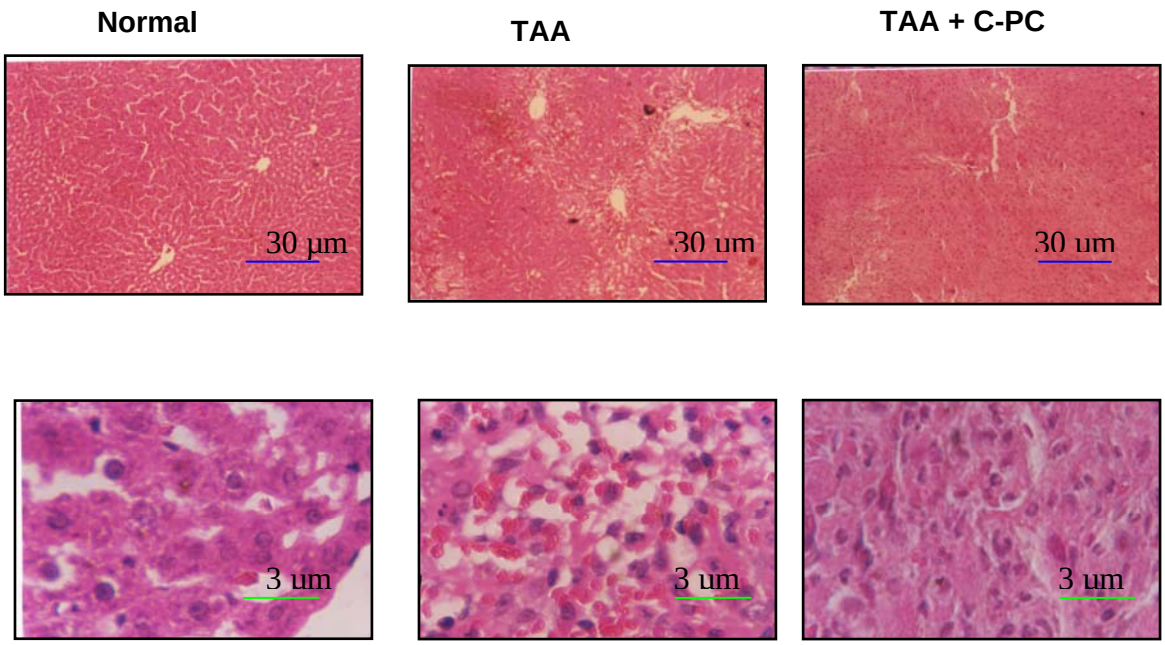


Fig: 5.2 Histopathological examination was performed on liver specimens obtained 18h after the administration of TAA + C-PC. The livers of rats treated with TAA alone exhibited significant necrosis and infiltration of neutrophils and mononuclear monocytes, The same was reduced in the TAA + C-Phycocyanin group.

Effect of TAA and C-PC on brain histopathology by light and electron microscopy.

Light microscopic images stained with toluidine blue revealed a marked reduction in cytoplasmic swelling of astrocytes in rats co-administered with C-PC (Fig 5.3).

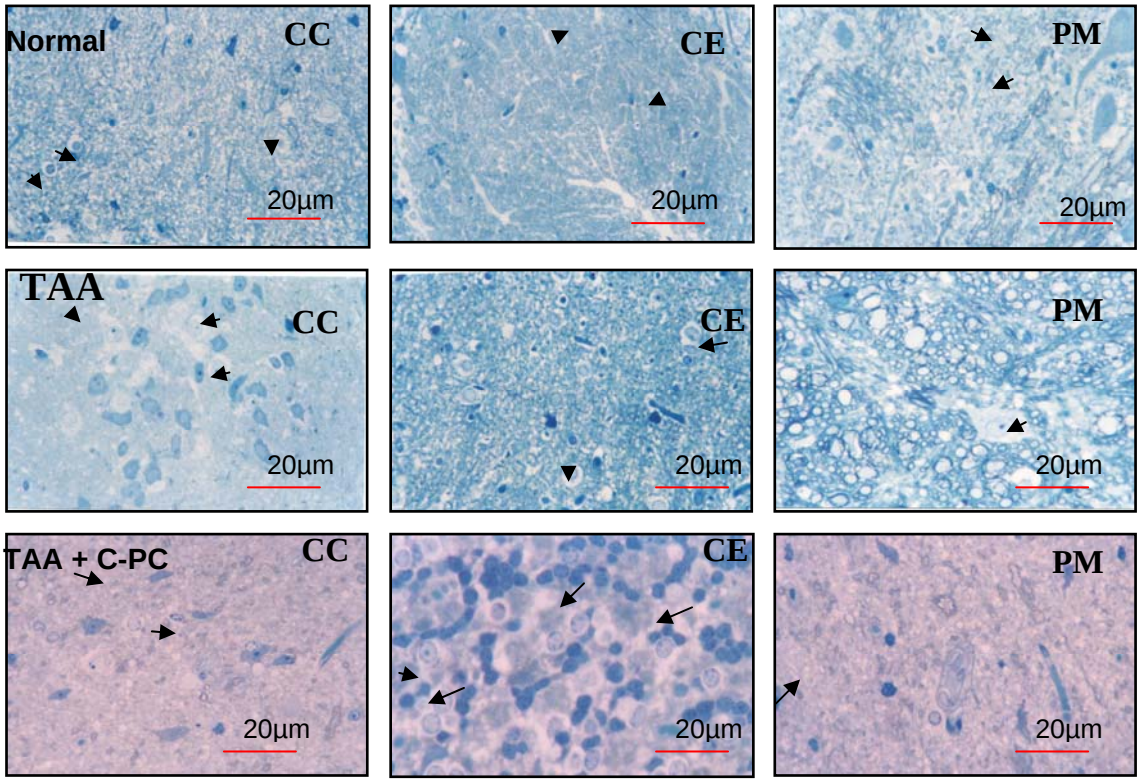
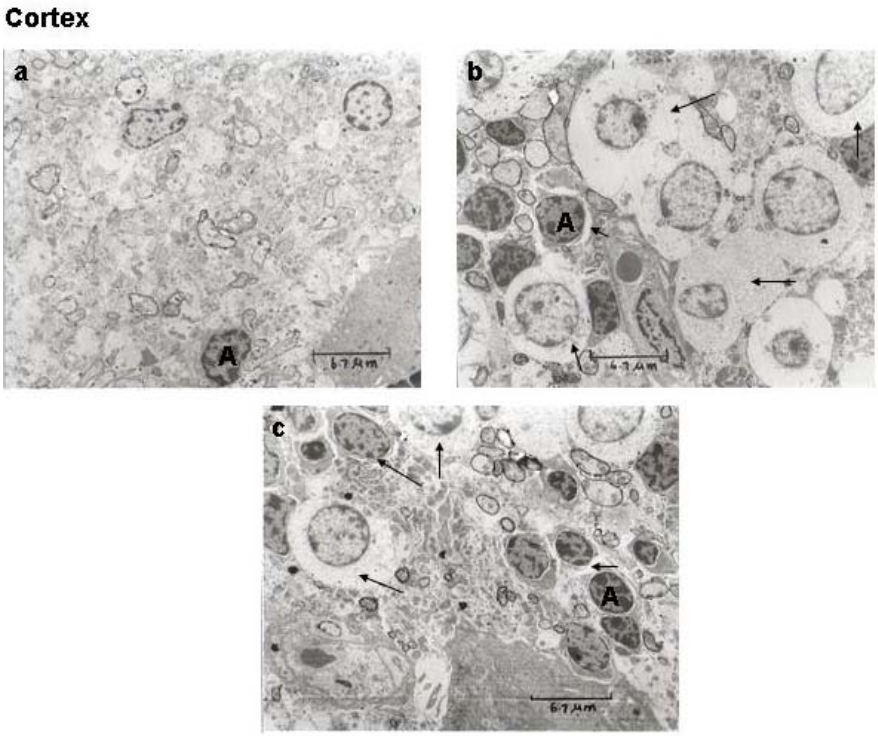


Fig: 5.3 Histopathological observations of different regions of brain stained with toluidine blue in normal, TAA treated and TAA+ C-Phycocyanin treated rats. Decreased astrocyte swelling was observed in different regions of brain in rats treated with TAA+C-Phycocyanin.
➔ İndicates astrocytes.

Electron Microscopic studies:



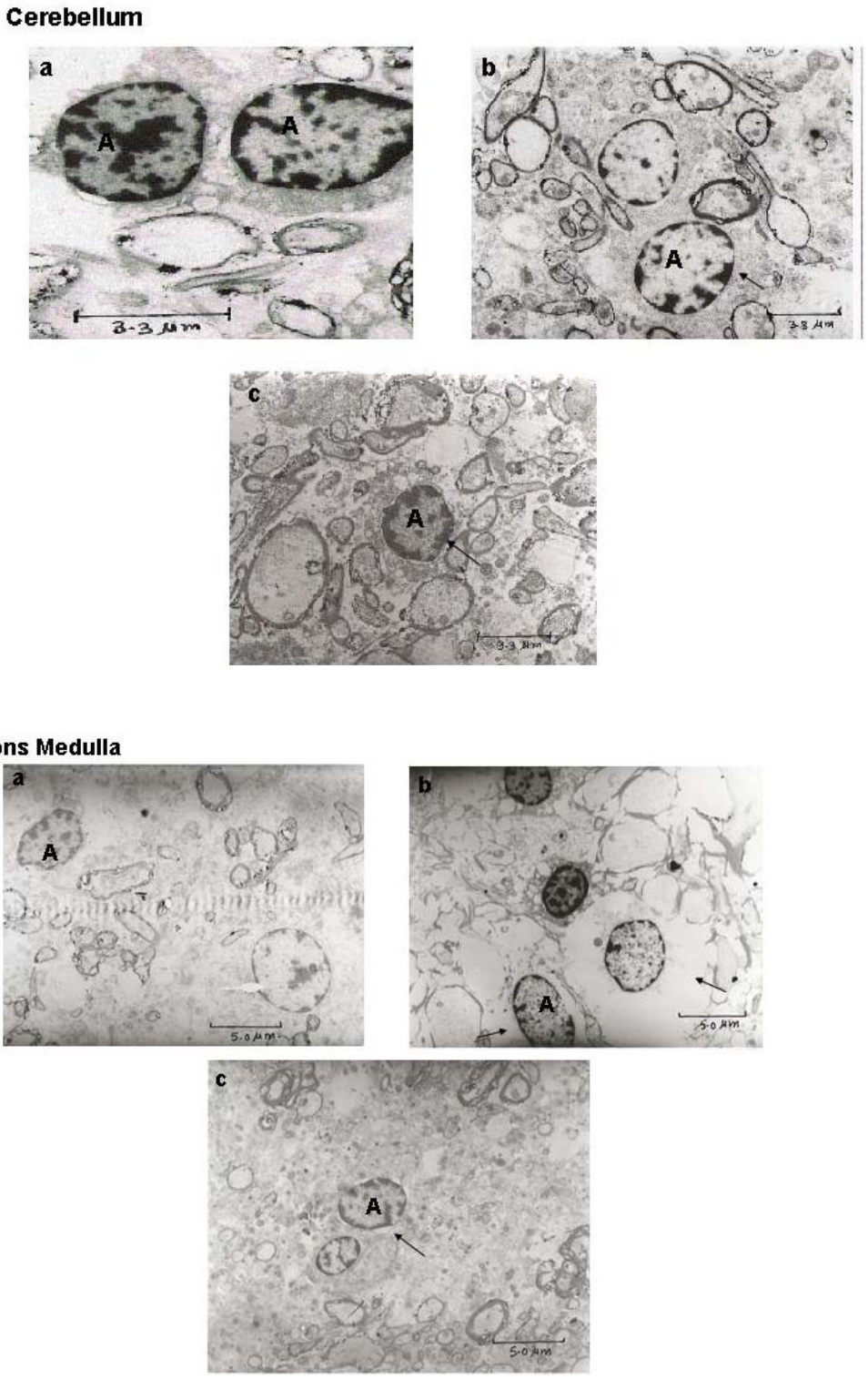


Fig: 5.4 Decreased cytoplamic swelling was observed in rats treated with TAA+C-PC in all the regions of brain. a) Normal b) TAA treated and c) TAA+C-PC treated. Arrow points the astrocyte swelling.

Brain MDA levels:

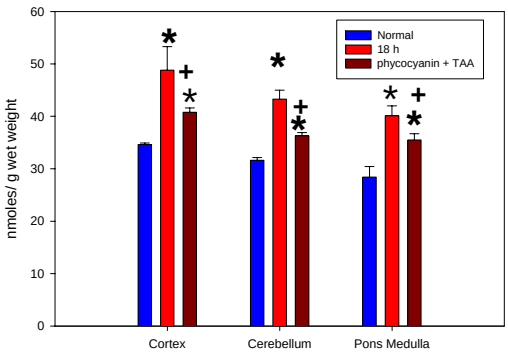


Fig: 5.5 The levels of MDA in cortex, cerebellum and pons medulla were, measured using the thiobarbituric method was significantly increased which received only TAA when compared to control. Co-administration of C-PC significantly reduced brain MDA levels. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.

Antioxidant defenses: Alterations in activities of Catalase and Glutathione peroxidase after TAA+C-PC treatment.

(A) (B)

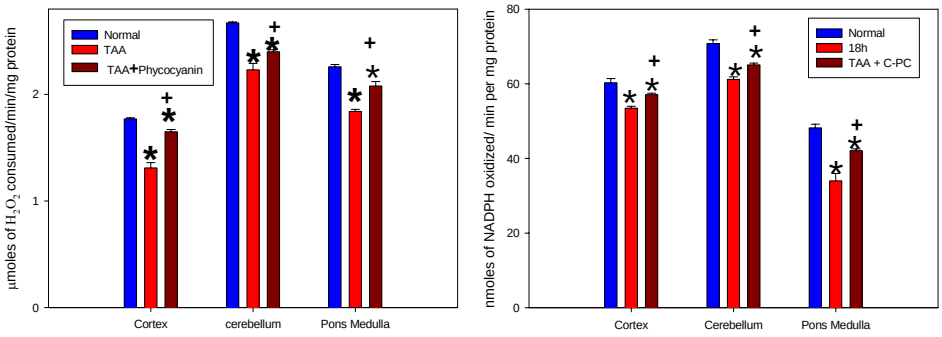


Fig: 5.6 The marked decrease in catalase (A) and glutathione peroxidase (B) activities observed in TAA treated rats was significantly attenuated by the treatment with C-PC. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control.+ indicates significant over TAA treated values.

Changes in the levels of total tryptophan in different regions of brain and serum.

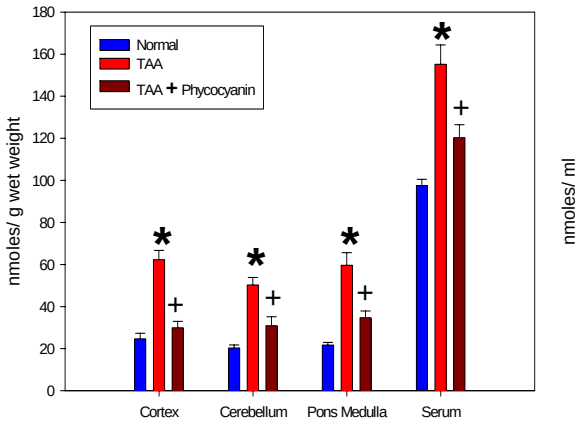


Fig: 5.7 A significant improvement was observed in tryptophan levels in both serum and various regions of brain after TAA+C-phycocyanin treatment. Values are mean ± S.E.M, n=3 done in duplicates. Statistical analysis was done using one way ANOVA by Student Newman-Keuls test. Level of significance was set at P< 0.05.* Significant over control + Significant over TAA treated values.

5.2 Discussion:

C-Phycocyanin is known for its various medicinal as well as pharmacological and nutritional properties (Farooq et al., 2004). C-PC was previously shown to be hepatoprotective in rats treated with carbon tetrachloride (Bhat and Madyastha, 2000). In the present study, the protective effect of C-PC was evaluated in rats with acute hepatotoxicity and HE induced by TAA. As mentioned in the earlier chapter, thioacetamide induced FHF in rats is similar to those reported earlier (Zimmermann et al., 1989, Larsen et al., 1994) and resembles human FHF form in most aspects and obeys Terblanche and Hickman (1991) conditions. Hence this model has been chosen to evaluate the efficacy of C-PC on TAA induced FHF.

The survival rate increased significantly following C-PC administration and most of the animals returned to normal activity by 10 days and continued to survive. Also these C-Phycocyanin co-administered rats showed marked improvement in liver function as evidenced by decreased serum AAT, AST, ammonia and decreased necrosis of the hepatocytes.

Oxidative stress is an important factor in the pathophysiology of HE. Free radicals can induce cellular damage via peroxidation of membrane lipids, breaking of DNA strands and denaturing cellular proteins (Maxwell, 1995). Reddy et al. (2004) have reported an increase in the activity of superoxide dismutase in the mitochondria of TAA induced rats. This would lead to accumulation of H_2O_2. H_2O_2 as such is more toxic than oxygen radicals and in the presence of transition metals like iron leads to the generation of the highly toxic hydroxyl ions which are known to induce lipid peroxidation (Harman, 1991). Since catalase and glutathione peroxidase are the main enzymes involved in detoxifying H_2O_2, their activity levels were measured. Also the rate of lipid peroxidation – a marker for oxidative stress, was measured with and without phycocyanin treatment.

Brain ammonia and MDA levels decreased significantly in TAA+C-PC treated rats compared to TAA alone treated rats. Cerebral edema and the associated increase in intracranial pressure are believed to be the main causes of death in patients with HE (Blei, 2005). Administration of phycocyanin caused a marked decrease in cellular edema, which was otherwise prominent in TAA treated rats. Thus phycocyanin could be used as a potential compound to

alleviate cerebral changes in HE, and thus prolong the survival time. In the clinical scenario this would help us gaining of valuable time for implementing other therapies for terminally-ill hepatic failure patients.

Administration of C-PC in the present study resulted in reducing the extent of liver damage considerably. This is evident from the liver histology and improvement in prothrombin time, serum albumin levels and significant decrease in liver and brain ammonia levels. The decrease in brain ammonia levels may be an important factor leading to improvement of cerebral conditions following C-Phycocyanin treatment as ammonia is believed to be the chief causative factor of HE during FHF. There was also a significant decrease in both brain and serum total tryptophan levels in rats treated with C-PC (Fig 5.7). The decrease in serum tryptophan levels is understandable because of a significant increase in the serum albumin levels and the decrease in brain could be due to the decreased transport of tryptophan to the brain. A reduction of elevated tryptophan levels achieved by C-PC could avoid an exaggerated production of quinolinic acid and serotonin. Since liver dysfunction is the main precipitating factor for HE, it remains to be ascertained whether the protective effect exerted by C-Phycocyanin on brain during HE is either direct or via alleviation of liver damage or both. A direct role for C-Phycocyanin on brain cannot be ruled out since it can effectively cross the blood brain barrier. Nevertheless, this is out of the purview of the present study. The present study, however, is aimed at addressing to the principal question of whether C-Phycocyanin prevents TAA-induced tissue damage and extends survival time or not. Though, phycocyanin

could not completely prevent the occurrence of liver dysfunction or its associated cerebral changes, it however, prolonged the survival time of rats treated with TAA. This increase in the survival rate indicates that CPC could facilitate recovery by prolonging the period between onset and mortality which in absence of intervention is very short for FHF.

The present study, thus effectively demonstrates that C-Phycocyanin at a dosage of 50mg/kg twice at 24h intervals of time prolongs the survival time in TAA induced FHF rats, with important consequences to the development of new therapeutic measures for HE. In addition, it also prevented the TAA-induced structural and functional derangements in the brain.

Further in-depth studies, however, are required to optimize the dosage and duration time and to understand the mechanism of action and study the long-term effects of the administration of C-PC.

Conclusion

Conclusion 6.0

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 neurotoxic, ultimately leading to death. In hepatic failure surveillance study, a very poor survival rate (22%) was observed in patients. Clinical outcome of FHF patients depends on the number of surviving hepatocytes, age, sex, etiology and the stage at which the patient is provided with medical assistance. No specific treatment or drugs are available for FHF and the outcome depends on the supportive care and better patient management.

In the present study, an animal model for FHF was developed in male Wistar rats using TAA (300 mg/kg), a hepatotoxin administered twice intraperitoneally at 24h interval. Analysis of biochemical parameters and liver function tests indicated impaired liver function. A seven fold (661%) increase in serum AAT was observed at 36h after the administration of TAA, while an eight fold decrease (81%) was seen in the activity of this enzyme in the liver tissue. Similarly, a three fold increase (218%) in the activity of serum AST was observed while a 10 fold decrease (90%) in the activity of this enzyme in liver. In addition, histopathological studies on liver revealed severe necrosis of the hepatic tissue and perivenular macrophage accumulation. The model also manifested typical symptoms of HE like stupor, defective tail pinch response and increased cerebral ammonia levels.

Having ascertained the reproducibility of this model and its resemblance to human HE, further studies were undertaken using this model.

Reports from literature suggest that hepatic encephalopathy is characterized by changes in sleep, mood, confusion and alterations in sleep patterns. Many of these functions involve neurotransmitters derived from tryptophan and its metabolites. Further, many of the neurodegenerative diseases like Huntington's chorea, Parkinson's and Aids-Dementia are also characterized by changes in cerebral tryptophan metabolism and its metabolites. As these tryptophan metabolites are potent free radical generators and lipid peroxidants, the present study is primarily aimed to analyze the serum and brain amino acid profiles, in particular by tryptophan.

The present study revealed marked increase in aromatic amino acids and glutamine in both serum and different regions of brain, cortex, cerebellum and pons medulla. However, there was no particular pattern of changes in branched chain amino acids in these regions. These results indicate enhanced accumulation of certain cytotoxic amino acids in different regions of brain specially tryptophan. Since tryptophan metabolites are potent lipid peroxidants, neuromodulators and free radical generators, further studies were undertaken on tryptophan metabolism.

Tryptophan is metabolized in brain in two important pathways namely, the serotonin and the kynurenine pathway. In the serotonin pathway, increase in both serotonin and 5-HIAA were observed, which could be responsible for the altered behavior and mood changes observed in animals treated with TAA. The increase

in serotonin levels was more or less uniform while 5-HIAA increase was more pronounced in pons medulla region. In the kynurenine pathway, increase was observed in anthranilic acid, 3-hydroxykynurenine and quinolinic acid. The levels of 3-hydroxykynurenine, a well known free radical generator, increased significantly in cortex after TAA administration. The changes in the kynurenine pathway might play an important role in affecting the red-ox state of the cell by generating oxidative stress as some of these metabolites are potent lipid peroxidants and free radical generators. Thus, it is evident that TAA-induced FHF causes alterations in both serotonin and kynurenine pathway metabolites.

A significant increase in the levels of malondialdehyde and nitric oxide were observed in different regions of brain, suggesting the induction of oxidative stress. Nitric oxide levels increased significantly in pons medulla and cortex regions. Lipid peroxidation and ROS levels also showed more increase in cortex and pons medulla when compared to cerebellum. Oxidative stress could be mediated by increased free radical generation or impaired antioxidant defense mechanism. A marked decrease was observed in the activity of the antioxidant enzymes catalase, glutathione reductase and glutathione peroxidase. Pons medulla and cortex showed a greater decrease in these activities when compared to cerebellum. These results suggest that cortex and pons medulla seem to be more affected than cerebellum. Thus, from the current study, it is clear that TAA-induced FHF leads to oxidative stress in different regions of brain as a result of increased generation of ROS coupled with impaired antioxidant defenses. This induced oxidative stress could result in tissue

damage, disruption of endothelial membranes and changes in blood brain barrier.

Ultrastructural studies on the brain tissues treated with TAA indicated cytoplasmic edema and mitochondrial swelling in astrocytes. Swelling was more pronounced in cortex and pons medulla as observed by light microscopy. This would have implications on the red-ox state of the cell and cellular metabolism. Astrocytes form the main metabolic support for neurons. Changes in astrocyte metabolism would adversely affect neuronal metabolism and functioning. Ultrastructural studies also showed changes in endothelial lining of cerebral small blood capillaries surrounded by astroglial endfeet, which could lead to the disruption of BBB. Further, studies on BBB involving intraperitoneal injections of Evans blue revealed enhanced damage to the BBB in the animals treated with TAA. Spectophotometric estimation of Evans blue and histopathological studies revealed a greater increase in pons medulla and cortex regions than cerebellum. These results infact confirm the impaired BBB, which might be responsible for observed increase in the level of ammonia and other amino acids in the brain tissue. It however, remains to be ascertained whether BBB endothelial changes occur during the initial phase of the disease progression or as a result of increased oxidative insults.

Since oxidative stress was found to be significant factor in cerebral changes during HE, the protective efficacy of C-PC, a hepatoprtotectant and antioxidant was probed. C-PC (50mg/kg) co-administered with TAA intraperitoneally twice at 24h caused a significant improvement in the survival of

rats. The rats treated with TAA+ C-PC survived beyond 10 days as against 20% survival upto 2 days in TAA alone treated rats. Besides survival rate, marked improvement in the liver and brain histology and decrease in lipid peroxidation levels was observed in rats treated with TAA+C-PC. The activity of liver enzymes AAT and AST improved significantly by 77% and 80% respectively while liver ammonia levels improved by 95% in rats treated with TAA+C-PC. Significant decrease in astrocyte swelling in different regions was observed suggesting decreased edema in phycocyanin co-administered rats. This study, therefore demonstrates the protective efficacy of C-Phycocyanin in ameliorating the changes associated with HE. However, further in-depth studies are required for standardizing the dosage and duration time to understand the therapeutic action for studying the long –term effects of administration of C-PC.

In summary, the present studies clearly demonstrate alterations of tryptophan metabolites during TAA-induced FHF as one of the main factors leading to oxidative stress. This induced oxidative stress results in changes in the histopathology of the brain and impairment in the permeability of the blood-brain barrier. C-Phycocyanin was able to ameliorate the toxicity of TAA induced changes in HE considerably, protecting the brain from structural and functional derangements. In the clinical scenario this would help gain valuable time for implementing other therapies for terminally –ill hepatic failure patients.

The overall mechanism involved in TAA-induced FHF and HE and the protection offered by C-PC is depicted in Fig 6.1.

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