Results & Discussion
3.1 Development of an Animal Model

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

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

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

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

The study of animal models for FHF has followed two different approaches.
1. Surgical procedures.
2. Pharmacological procedures

3.3.1 Animal Models Based on Surgical Procedures

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

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

3.3.2 Animal Models Based on Pharmacological Procedures

<table>
<thead>
<tr>
<th>Model</th>
<th>Animal</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D- Galactosamine</td>
<td>Rats</td>
<td>Encephalopathy, Increased AST, ammonia, hepatic necrosis.</td>
<td>Kepler et al., 1968</td>
</tr>
<tr>
<td></td>
<td>Rabbit</td>
<td></td>
<td>Blitzer et al., 1978.</td>
</tr>
<tr>
<td></td>
<td>Dogs</td>
<td></td>
<td>Diaz Buxo et al., 1997.</td>
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<tr>
<td></td>
<td>Pigs</td>
<td></td>
<td>Miller et al., 1976.</td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>Rats</td>
<td>Late stage coma</td>
<td>Shi et al., 1998.</td>
</tr>
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</table>
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The different pharmacological agents sited above have different modes of action for the induction of FHF. The potency and the reproducibility vary with different animals.

3.4 Thioacetamide-Toxicity

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

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

The feeding of the animal decreased considerably after the first dose of TAA and is stopped completely during 12-24 h after its second dose. Moreover the animal became progressively inactive and sleepy...
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from 6 h after the administration of the second dose. From 12 h after the second dose, these animals showed wobbly gait and loss of Tightening reflexes. This change in the gait and loss of reflex was enhanced after 18 h after the second dose of thioacetamide.

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

3.5 Liver Function Tests
3.5.1 Glucose

Liver is a vital organ that plays a key role in the homeostasis of blood composition, specifically blood glucose levels. So any damage to the liver will be reflected directly in the blood glucose levels. Hence, to assess the liver damage in thioacetamide toxicity, glucose levels were estimated in serum as well as in the liver of normal and drug administered rats at different time intervals. In the present study, serum glucose levels were determined instead of whole blood glucose levels. This was done keeping in mind the fact that blood has different types of cells which also trap glucose. This glucose is used exclusively by these blood cells for their metabolic demands and is not available for other tissues/cells whereas the glucose present in the serum is available not only to blood cells but also to cells in other organs.
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The glucose levels were decreased in both serum (Fig. 1a) and liver (Fig. 1b) after the administration of thioacetamide. In liver the glucose level was decreased by 50% of the control value within 12 h after administration of thioacetamide and by 36 h the glucose level in liver was decreased by 75%.

The fall in the serum glucose level was not so rapid as compared to that of liver. The magnitude of decrease in serum glucose levels was more or less same (-20%) as that of liver in the initial stages of toxicity. But by 12 h, the decrease in liver glucose level was much higher than those in the serum. At the end of 36 h, serum glucose levels were only half while that of liver was one fourth of the respective control values. The decrease in serum and liver glucose levels might be due to two reasons - liver damage caused by thioacetamide and decreased feeding of the animal. As the animal stops feeding, naturally blood glucose levels would decrease rapidly. Under such conditions, in normal animals, liver would maintain blood glucose levels initially by glycogenolysis and later by gluconeogenesis. However, in the drug treated animals, this process may not be operative in an effective manner due to the hepatotoxic effects of thioacetamide (Wagle et al., 1976; Hoyumpa and Schenker 1985).
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Fig. 1: Levels of glucose in the (a) serum and (b) liver of normal and thioacetamide treated rats at various time points

* Significant over control
† Significant over previous time point
3.5.2 Ammonia

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

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

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

* Significant over control
† Significant over previous time point
Hence, the ammonia should have been produced from internal sources. One such source is liver where amino acids are utilized for the production of glucose through the pathway of gluconeogenesis resulting in the production of ammonia. In addition, intestinal smooth muscle is known to preferentially use glutamine as the source for its energy and this also results in the production of ammonia. In addition, failure of mechanisms to remove ammonia in the body would also contribute to increase in blood ammonia levels. As liver is the major site of ammonia removal in the body, ammonia levels in the liver were measured and more or less changes similar to those in serum were observed in liver (Fig. 2 c). This increase in liver ammonia level might be due to decreased detoxification of ammonia to urea in this tissue due to metabolic derangements leading to necrosis of liver cells. Hence, in thioacetamide induced liver failure, ammonia that enters the liver escapes the detoxification process and large amounts of ammonia enters the systemic circulation. The increase in liver ammonia level is known to be reflected in elevated serum ammonia levels in liver failure conditions (Bruck et al., 1999; 2002). In the present study also the changes in liver ammonia levels are faithfully reflected in the serum. This is supported by the correlation coefficient of 0.84 between liver and serum ammonia levels (Fig. 3 b). Once ammonia enters systemic circulation, it will flood the tissue such as brain. Such an elevation in the brain ammonia levels is well known to be a hallmark of this condition (Albrecht et al., 1990; Norenberg 1998).
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Fig. 3: Correlation between (a) serum-brain (b) serum-liver ammonia levels in rats with TAA-induced FHF
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Results of the present study also indicate a progressive increase in brain ammonia levels in thioacetamide induced fulminant hepatic failure (Fig. 2b). Moreover, a close correlation ($r^2 = 0.97$) was seen between serum and brain ammonia levels (Fig. 3a). Increased levels of ammonia in brain has been considered as the chief culprit in the neurological dysfunction observed in fulminant hepatic failure (Albrecht 1998; Butterworth et al., 1987; Norenberg 1996; 1998).

3.5.3 Urea

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

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

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

### 3.5.4 Proteins

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

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

a. SERUM PROTEIN

![Graph of serum protein levels over time]

b. LIVER PROTEIN

![Graph of liver protein levels over time]

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

3.5.5 Aminotransferases

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

a. SERUM ALT

b. LIVER ALT

* Significant over control
↑ Significant over previous time point
Fig. 7: 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
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Fig. 8: Correlation between
(a) Serum and liver alanine aminotransferase and
(b) Serum and liver aspartate aminotransferase in thioacetamide treated rats

![Graphs showing correlation between serum and liver enzymes.](image)
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The increase in the activities of these enzymes in the serum might be due to the necrosis and subsequent release of these enzymes from the hepatocytes into the blood.

3.5.6 Prothrombin Time

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

The clotting of blood depends on the primary platelet plug formed along with the formation of a stable fibrin clot. Liver is the primary site of production of Fibrinogen and Prothrombin. The latter factor plays a crucial role in initiating the cascade of blood clotting reactions. Prothrombin is converted to thrombin in the presence of Ca^{++} and thrombin converts fibrinogen to fibrin.
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Fig. 9: Changes in the prothrombin time in normal and thioacetamide treated rats

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

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

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

Results of the present study clearly established the potency of thioacetamide in inducing fulminant hepatic failure conditions in rats.
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Having established the animal model for FHF, attention is now focused on the involvement of the nonsynaptic mitochondria in the pathophysiology of the disease. For this purpose, contamination-free nonsynaptic mitochondria were isolated by density gradient centrifugation and used for studying the role of cerebral mitochondrial dysfunctions in the etiology of FHF.
Fig. 10: Photomicrographs showing the liver cells from the control rats. These cells show the presence of normal hepatocytes with distinct nucleus. a : Lower magnification (30 μm = 1.025 cm); b : Higher magnification (10 μm = 1.5 cm)
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**Fig. 11**: Photomicrographs of liver taken from the rat after 6 h thioacetamide treatment. The micrograph shows the presence of necrotic (degenerating) hepatocytes, which are mainly concentrated around the capillaries (blood vessels). (→) shows the presence of a capillary. Fairly condensed nuclei that are darkly stained in the necrotic hepatocytes while the surrounding cells, which are away from the damaged region, still show large spherical nucleus. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)
Fig. 12: These micrographs show the prolonged effect of thioacetamide on liver cells after 12 h after the treatment with the drug. The presence of big patches of degenerating cells with highly condensed nucleus in the hepatocytes are noticed here. These patches are present near the capillary (→) and also away from the capillary indicating the increased area of liver degeneration. The changes in the nucleus shape are clearly noticed. Lower magnification (30 µm = 1.025 cm); b: Higher magnification (10 µm = 1.5 cm)
Fig. 13: Micrographs showing the effect of thioacetamide at the end of 18 n of treatment after the second dose. With the longer treatment of thioacetamide there was a significant increase in the degeneration of the liver. Due to the higher degree of necrosis, well-condensed nuclei are observed in this preparation. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)
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Fig. 14: The micrographs here show the highest degree of damage done to the liver at the end of 24 h after the treatment with the drug. Almost all the liver cells are degenerated. Maximal condensation of the cellular mass apart from the condensation in the nucleus can be seen. It is difficult to find normal hepatocytes at this time point after the drug treatment. Lower magnification (30 μm = 1.025 cm); b: Higher magnification (10 μm = 1.5 cm)
TAA-induced FHF: Effects on cerebral nonsynaptic mitochondria
4.1 Standardization of the Enzyme Assays

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

4.2 Preparation of Nonsynaptic Mitochondria

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

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

4.3 Assessment of the Purity of the Isolated Subcellular fractions

In order to carry out the studies involving mitochondria, a subcellular fraction, it is very much necessary to determine the purity of the isolated fraction and the extent of cross contamination with other subcellular fractions. Usually microscopic and biochemical methods are used for the assessment of the purity of the fractions obtained. Electron microscopy is generally used since the resolution of the light microscope is not adequate to study the subcellular fractions. This procedure involves large amount of time in processing of the sample
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and thus it restricts the regular usage of electron microscope for the determination of the purity. Hence biochemical methods which have been well developed have been used in the present study. For this purpose, activities of the marker enzymes, whose subcellular localization have already been established were assessed. The activity of Succinate dehydrogenase (SDH), an enzyme located in the inner mitochondrial membrane was used as a marker for the assessment of the mitochondrial purity (Singer et al., 1972). Glutamic acid decarboxylase (GAD) was used as a marker for the synaptosomes (Salganicoff and De Robertis, 1965). The mitochondria present in these synaptosomes are called synaptic mitochondria. In the present study the term mitochondria refers to the one that sediments independent of the synaptosomes, which are referred to as non-synaptic mitochondria.

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

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

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

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

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

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

Similar results were obtained with the ficoll-400 based isolation of subcellular fractions. When this method was adopted synaptosomes
Activity of (a) succinate dehydrogenase (b) glutamic acid decarboxylase in different subcellular fractions (myelin, synaptosomes, and mitochondria) isolated by sucrose density gradient. For SDH marker, its activity was taken as 100% in mitochondria and the activity of other fractions was expressed as relative % activity of mitochondria. For GAD marker, activity of GAD in synaptosomes was considered as 100% and the activity of other fractions was expressed as relative % activity of synaptosomes.
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Fig. 16: Assessment of purity of the subcellular fractions isolated by Ficoll-400 density gradient

Activity of (a) succinate dehydrogenase (b) glutamic acid decarboxylase in different subcellular fractions (myelin, synaptosomes, and mitochondria) isolated by Ficoll-400 density gradient. For SDH marker, its activity was taken as 100% in mitochondria and the activity of other fractions was expressed as relative % activity of mitochondria. For GAD marker, activity of GAD in synaptosomes was considered as 100% and the activity of other fractions was expressed as relative % activity of synaptosomes.
had the highest activity while mitochondria had 11% and myelin had 31% of the activity of synaptosomes (Fig. 16b) indicating that all the three fractions are being isolated separately without getting co-separated with the other fractions.

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

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

4.4.1 Citric Acid Cycle Enzyme Activities

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

The activity of pyruvate dehydrogenase (PDH) was determined by following the decrease in absorbance at 340nm. The activity of Pyruvate dehydrogenase showed a decrease of 8.2% by the end of 12 h and then by 20.7% by 18 h. At the end of 24 h the PDH recorded its lowest activity with a decrease of 36% over the control (Fig. 17a). The activity of Citrate synthase did not show any significant alterations in its
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activity at any of the time periods after the administration of the drug. Though there was an initial increase followed by a decrease, the change was not statistically significant (Fig. 17b). The activity of Isocitrate dehydrogenase did not show any statistically significant change in its activity, though there was a slight increase at 18h interval (Fig. 18a). At 18 h the activity of a-ketoglutarate dehydrogenase was elevated by 34.7% and at 24h the increase in its activity was 27% (Fig. 18b). The change in the activity at 18 h was significant over both control as well as over the previous time point. Succinate dehydrogenase recorded a significant decrease in the activity at all the time points after the administration of the drug. This enzyme showed a reduction of 15% and 31% over the control at 12 and 18 h respectively (Fig. 19a). At 24 h the decrease was 23% in the activity of SDH. Malate dehydrogenase, in the direction of oxaloacetate formation, did not show significant change in the activity at all the time periods studied after the thioacetamide administration (Fig. 19b). The activity was more or less the same at all the time periods of treatment.
Changes in the activities of (a) pyruvate dehydrogenase and (b) citrate synthase in the nonsynaptic mitochondria isolated from the cerebral cortex of normal and FHF rats at 12, 18 and 24h after thioacetamide administration.

* Significant over control. † Significant over previous time point
Fig. 18: Activities of isocitrate dehydrogenase and α-ketoglutarate dehydrogenase

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

* Significant over control. † Significant over previous time point.
Fig. 19: Activities of Succinate dehydrogenase and Malate dehydrogenase in brain mitochondria

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

* Significant over control.
4.4.2 Effect of Thioacetamide-Induced FHF on the Activities of Electron Transport Chain Enzymes

4.4.2.1 NADH - Ubiquinone Oxidoreductase (Complex I)

The decrease in the absorbance due the oxidation of NADH at 340nm leading to the reduction of ubiquinone (CoQ1) to ubiquinol was measured. The activity of Complex I showed a decrease of 31% at 18h and 21% at 24 h in the thioacetamide treated rats in comparison with normal rats (Fig. 20a).

4.4.2.2 Succinate - Ubiquinone Oxidoreductase (Complex II)

The disappearance of the colour of the DCPIP dye due to the secondary reduction of the dye by the ubiquinol that is formed as a result of the reduction of the ubiquinone (CoQ2) compound was measured at 600nm. The activity of complex II showed no significant alteration in the experimental rats when compared to those in control rats (Fig. 20b).

4.4.2.3 Ubiquinone-Cytochrome-c Oxidoreductase (Complex III)

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

The activity of Cytochrome-c oxidase was determined by monitoring the decrease in the absorbance at 550nm due to the oxidation of cytochrome c. Before the reaction was done, cytochrome c was reduced by the addition of a pinch of ascorbate. No significant differences were observed in the activity of respiratory complex IV at any of the time periods after the induction of FHF (Fig. 21b).
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Fig. 20: Effect of thioacetamide-induced FHF on NADH-ubiquinone oxidoreductase and Succinate-ubiquinone oxidoreductase

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

* Significant over control. † Significant over previous time point
Fig. 21: Effect of thioacetamide-induced FHF on Ubiquinone-cytochrome c oxidoreductase and Cytochrome c oxidase

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

* Significant over control. † Significant over previous time point
4.4.3 Measurement of Oxygen Consumption Studies in the Non-synaptic mitochondria.

**Polarographic Method**

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

The results of this study indicated that the changes in the mitochondrial respiration, when succinate was used as a substrate, were not statistically significant. An ideal curve indicating various states of the respiration has been shown in the figure (Fig. 22). In a typical assay, the reaction mixture contained sucrose, mannitol, KH$_2$PO$_4$, KCl, EDTA, and Tris-HCl, and the mitochondrial sample. The reaction was initiated by the addition of the substrate (succinate or pyruvate and malate). ADP was added to reaction mixture to measure the state 3 respiration and P/O ratio. The ratio of state 3 to state 4 was taken as the respiratory control ratio. The P/O ratio was calculated as the amount of ADP to that of the oxygen consumed during the state 3 respiration.
Fig. 22: A typical polarographic curve showing different stages of mitochondrial respiration

The results of this experiment indicated that there was no change in the state 3 and state 4 respiration when succinate was used as the respiratory substrate (Fig. 23a, 23b). Hence no change was observed in the respiratory control ratio and in the P/O ratio (Fig. 23c, 23d). On the other hand, the state 3 respiration was lowered by 27% when Pyruvate–malate were used as the substrates (Fig. 23a). The respiratory control ratio was also found to be reduced by 22% in the mitochondria of the drug administered rats when pyruvate/malate were
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used as the substrates (Fig. 23c). Though the P/O ratio showed a
decrease in pyruvate/malate induced respiration, it was not statistically
significant (Fig. 23d).

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

Mitochondrial Swelling

In order to see if there is any change in the structure of the
mitochondria, changes in the volume of the mitochondria were
determined by measuring the swelling of the mitochondria. The results
of this study indicate that the volume of the mitochondria was
significantly increased in the thioacetamide treated rats. The swelling
studies performed by following the decrease in absorbance revealed a
two fold increase in the volume in mitochondria (Fig. 24). This increase
in the volume was further confirmed by the transmission electron
microscopy. The photomicrographs of the TEM quite evidently reveal
the changes in the mitochondria of the FHF induced rats (Fig. 25). The
increase in the volume of mitochondria in the TAA-induced FHF rats
(Fig. 25b) over the control (Fig. 25a) is indicated by the arrow mark.
The magnified image of the mitochondria of Control (Fig. 25c) and drug
administered rats (Fig. 25d) is also shown.
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Fig. 23: Effect of thioacetamide induced FHF on mitochondrial respiration

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

* Significant over control.

Changes in different parameters of the nonsynaptic mitochondrial respiration,
Mitochondrial swelling was assessed by measuring the change in absorbance at 540nm in the nonsynaptic mitochondria of normal and FHF rats, 18 h after the administration of thioacetamide. * Significant over control.
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Fig. 25: Transmission electron micrographs showing the swelling of mitochondria

Transmission electron micrographs of ultra thin sections of cerebral cortex of thioacetamide induced FHF rats at 18 h after the administration of the drug showing the volume changes in the mitochondria of normal (a & c), TAA induced FHF (b & d)
4.6 FHF Induced Oxidative Stress in the Mitochondria From Cerebral Cortex of Rats

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

In the experimental animals with induced FHF, there was an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the nonsynaptic mitochondrial lipid peroxidation (Fig. 26). There was an increase in lipid peroxidation by 46% as early as 12 h after thioacetamide administration. At 18 h, it further increased by 140%, and then by 180% at 24 h after the administration of thioacetamide. The levels of total thiols, on the other hand, decreased by 14% at 12 h interval and 30% by 18 h when compared with the controls (Fig. 27). At 24 h, no further change was observed. There was no significant change in the activity of glutathione peroxidase at 12 h interval but there was a statistically significant decrease of 14% and 24% at 18 and 24 h, respectively (Fig. 28). The activity of glutathione reductase also decreased by 17% over the
controls, but only at 24 h (Fig. 29). There was an increase of 39% and 46% in the activity of Mn-SOD at 18 and 24 h after the administration of the thioacetamide, respectively (Fig. 30).

Further there was an increase of 44% in the levels of the reduced glutathione (GSH) content in the thioacetamide injected rats (Fig. 31). The oxidized form of glutathione (GSSG) was elevated by 2.7 folds in the TAA-induced FHF rats (Fig. 32). As a result of the changes in the GSH and GSSG levels the ratio of the GSH/GSSG was decreased by 52% in the rats affected with FHF (Fig. 33).
Fig. 26: Malondialdehyde levels in the brain mitochondria of FHF rats

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

* Statistically significant over the control
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Fig. 27: Levels of total thiols in the brain mitochondria of FHF rats

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

* Statistically significant over the control.
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Fig. 28: Effect of thioacetamide-induced FHF on glutathione peroxidase activity

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

All values are mean ± Standard deviation of six individual experiments done in duplicates.

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

* Statistically significant over the control.
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Fig. 31: Changes in the content of GSH in nonsynaptic mitochondria of normal and thioacetamide-induced FHF rats

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

* Statistically significant over the control.
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Fig. 32: Changes in the content of GSSG in normal and thioacetamide-induced FHF rats

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

* Statistically significant over the control.
Fig. 33: Changes in the GSH/GSSG ratio of normal and thioacetamide-induced FHF rats

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

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

In the present investigation, a thioacetamide induced FHF rat model was developed and characterized. Results of the work that has been discussed in previous chapter clearly established the induction of fulminant hepatic failure by the administered dose of thioacetamide into the rats. Having established the rat model for FHF, attention was focused on to the involvement of the nonsynaptic mitochondria in the pathophysiology of the disease. The isolation of non synaptic mitochondria from brain specimens is complicated by the presence of myelin and synaptosomes which co sediment with mitochondria in normal differential centrifugation. In order to avoid the contamination of the mitochondria with the synaptosomes and with the vesicles formed by the sheared nerve endings during homogenization, metabolically active contamination-free nonsynaptic mitochondria were used in the present study. Non synaptic mitochondria from the cerebral cortex of adult Wistar rats were isolated by following the method of Cotman et al., (1974) as described by Ratnakumari and Murthy (1989).
Pathological concentrations of ammonia are known to affect cerebral energy metabolism either by draining away the intermediates of TCA cycle for ammonia detoxification or by depleting cerebral energy reserves. Though there are reports on changes in intermediates, very little information is available on the activities of the enzymes involved in the energy metabolism such as reactions of the TCA cycle and electron transport chain complexes. Another possibility is that there may be uncoupling of oxidative phosphorylation leading to decreased energy production and mitochondrial damages. Hence the changes in the activities of the citric acid cycle enzymes were measured along with the alterations in the electron transport chain complexes in the non-synaptic mitochondria of thioacetamide induced FHF.

The activity of the pyruvate dehydrogenase (PDH) was lowered in the mitochondrial samples at 18 and 24 h of the thioacetamide treated rats. This decrease in the PDH activity would retard the channeling of pyruvate into TCA cycle. Citrate synthase activity which was measured by determining the formation of 5-thio-2-nitrobenzoate due to the coupling of CoA with DTNB at 412nm showed no change in the activity. Activity of citrate synthase in vivo depends on the availability of Acetyl Co A and oxaloacetic acid. The increase in α-ketoglutarate dehydrogenase activity observed in the present study would rapidly decarboxylate 2-oxoglutarate to succinate and thus pulls the isocitrate dehydrogenase reaction forward. Lack of change in
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citrate synthase and elevation in $\alpha$-ketoglutarate dehydrogenase would pull the isocitrate dehydrogenase reaction forward. Further, a decrease in the activity of succinate dehydrogenase (SDH) was observed in the drug administered FHF rats. Decrease in the activity of SDH might also alter the oxidation of succinate and thus the furtherance of the TCA cycle. An increase in $\alpha$-ketoglutarate dehydrogenase activity and a decrease in SDH activity could lead to the accumulation of succinate, which leads to a subsequent decrease in rate of formation of malate and oxaloacetate. These changes in TCA cycle may have impact on synaptosomes also as synaptosomes also contain mitochondria.

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

The activities of complex I and III showed a significant decrease while complex II and IV remained unaltered in the thioacetamide induced hepatic failure rats. A decrease in the activity of complex I and III may lead to an elevation in rate of free radical production through ETC. Similar elevation in the generation of ROS as a result of the complex I and complex III was reported in sub mitochondrial particles.
(Paradies et al., 2001; Bayer et al., 1992). Further, reports have also shown that the loss of these complex activities was prevented by the addition of exogenous free radical scavengers (Cardoso et al., 1999; Paradies et al., 2001). The inhibition of the complex III activity might be due to the loss of the mitochondrial membrane cardiolipin content as a result of the peroxidation of the membrane cardiolipin (Paradies et al., 2000; 2001). This effect would lead to the leakage of electrons from the ETC and thus generating more of superoxide radicals and hence more free radical induced damage to the mitochondria. Inhibition of Complex III has been reported to lead the production of superoxide radicals (Cardoso et al., 1999). The superoxide radicals will be dismutated to hydrogen peroxide, which in turn will react with superoxide radicals to produce more potent hydroxyl radicals. These hydroxyl radicals are even more devastating than the superoxide radicals. The enhanced production of these superoxide, \( \text{H}_2\text{O}_2 \) and hydroxyl radicals has been known to promote mitochondrial membrane alterations (Cardoso et al., 1999). Moreover, the reaction of super oxide anion radicals increase the auto oxidation of the ubisemiquinones, leading to the enhanced release of the electrons from the ETC (Benzi et al., 1991). Since brain demands high energy requirements, the inhibitions of the respiratory complex activities might have a pronounced effect on the functioning of the brain which could have an etiology in the pathophysiology of the disorder. Further complicating the situation are observations that indicate that free radicals can primarily affect complex I and III leading
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to a decrease in their activities thus completing the vicious cycle. However, it remains to be established which of these two events precedes the other.

One of the possible mechanisms involved in the hyperammonemic or FHF conditions may be associated with the increased production of the reactive oxygen species (Dugan et al., 1995; Piantadosi and Zhang, 1996). In such conditions studies have demonstrated the occurrence of depletions in the ATP levels in the brain (Hindfelt et al., 1973; Kosenko et al., 1994; Kosenko et al., 1993; McCandless et al., 1981) which suggest that there could be changes in the mitochondrial respiration. For this purpose we studied the rates of different states of respiration in the mitochondria isolated from the TAA-induced FHF rats. The data indicate a decrease in the state 3 respiration of the mitochondria when pyruvate/malate was used as the substrate. This may be an indication for the lowered functional efficiency of the mitochondria in terms of the respiration. The decrease in the respiratory control ratio, when pyruvate/malate was used as the substrate might probably be due to the loss of integrity of the mitochondrial membrane. Similar findings were reported by Gracia et al., (2003) and Kosenko et al., (1997a) in the experimental conditions of ammonium acetate administered rats. Hindfelt and his group (Hindfelt et al., 1977) have suggested that the defect in hyperammonemonic conditions may be due to the inhibition of malate-aspartate shuttle which reduces the transport of redox equivalents to
the mitochondria. In support of this alterations in cytosolic and mitochondrial NADH/NAD ratios have been reported in brain in hyperammonemic states. The decrease in the P/O ratio however, was not statistically significant. But when it is considered over a large time period it might result in a significant decrease in the ATP synthesis.

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

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

Increased NMDA receptor activity in hyperammonemic conditions (Marcaida et al., 1992; Hermenegildo et al., 1996) is reported to be an effector of excitotoxic neuronal death (Beal, 1992). Elevated NMDA receptor activity results in increased Ca\(^{++}\) influx (Kosenko et al., 1997; White et al., 1996) which would hamper the ATP
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synthesis and thus mitochondrial membrane potential. This might further result in mitochondrial electron transport chain alterations leading to enhanced production of free radicals (Choi, 1996; Nicotera et al., 1997).

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

In the present study we have also evaluated the involvement of oxidative stress on non synaptic mitochondria isolated from cerebral cortex in conditions of hepatic encephalopathy. This was done by studying changes in lipid peroxidation, total thiol and various antioxidant enzymes after inducing FHF by administering thioacetamide. A better understanding of the role of mitochondria in generating the ROS as well as the consequences of oxidative stress will surely contribute to the existing knowledge on FHF disorder. Assays on SOD, GPx, GR, MDA, total thiols chosen in the present study will conveniently evaluate the role of oxidative stress, if any, in the pathophysiology of FHF. These studies resulted in an increase in the levels of malondialdehyde (MDA) indicating a very significant enhancement in the nonsynaptic mitochondrial lipid peroxidation. There was an increase in lipid peroxidation by 46% as early as 12h after thioacetamide administration. At 18h, it was further increased by 140%, and then by 180% at 24h after the administration of thioacetamide. The levels of total thiols, on the other hand, were decreased by 14% at 12h interval and 30% by 18h when compared with the controls. There was
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no significant change in the activity of glutathione peroxidase at 12h after thioacetamide administration but decreased significantly by 14% and 24% at 18 and 24 h respectively. The activity of glutathione reductase also decreased by 17% over the controls, but only at 24 h. There was an increase of 39% and 46% in the activity of Mn-SOD at 18 and 24 h respectively after the administration of the thioacetamide.

Thus the studies on thioacetamide-induced FHF in rats reveal the induction of oxidative stress in nonsynaptic mitochondria as evidenced by the increased lipid peroxidation, decreased thiols and impaired antioxidant defenses. Oxidative stress is a condition in which the production of free radicals is far in excess of their rate of detoxification by endogenous mechanisms (Rao, 2002). Being a highly aerobic tissue accounting for 20% of total oxygen consumed by the body, brain is prone and vulnerable for oxidative stress. Free radicals are produced in the normal course of respiration and is estimated to be about 1-3% of the total oxygen consumed by the tissue (Kowaltowski et al., 1990). Furthermore, brain is rich in polyunsaturated fatty acids (Halliwell, 1992) and possesses high content of iron in certain areas, which is supposed to promote free radical production. Added to this, brain has low levels of antioxidant enzymes, low repair mechanisms and non-replicative neuronal cells (Halliwell, 1992). All the aforesaid factors play a critical role in balancing the oxidative stress and the antioxidant defenses. Mitochondria are the major sites of production of free radicals especially by the respiratory electron transport chain
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(Chance et al., 1979). Under normal physiological conditions the free radicals produced are detoxified by a variety of endogenous free radical scavengers such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase etc. In the present condition of induced FHF, there is enhanced production of free radicals leading to lipid peroxidation coupled with impaired antioxidant defenses in nonsynaptic mitochondria, probably leading to their damage.

One of the major consequences of oxidative stress is lipid peroxidation. The free radicals oxidize the fatty acids present in the membranes leading to the production of lipid peroxides. Continuous increase in the formation of nonsynaptic mitochondrial malondialdehyde observed in the present study suggests that the peroxidation starts as early as 12 hours to reach a maximum at 24h time period. Lipid peroxidation is a chain reaction unless a check is imposed by antioxidant defenses. Impaired antioxidant defenses will increase the levels of peroxides, which eventually damage the membrane vesicles by altering the physicochemical properties of the membrane (Paradies et al., 2001). Further the loss of the integrity of the mitochondrial membrane leads to mitochondrial dysfunctions, more precisely, respiration and oxidative phosphorylation (Masini et al., 1985). Increase in the lipid peroxidation implies elevated production of free radicals vis-à-vis increased ROS to impose an inhibition on respiratory electron transport chain complexes (Masini et al., 1985).
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Mn-SOD is an important mitochondrial antioxidant enzyme and its activity in the present study was elevated in a time dependent manner after inducing FHF. Kosenko et al., (2003) on the other hand, reported decreased Mn-SOD activity after the injection of ammonium acetate in rats. The apparent discrepancy in our results is not clear at the moment. Nevertheless, administration of ammonium acetate will create acute toxicity right away where as thioacetamide-induced FHF will be counter-acted by the system, which might have apparently reflected in the elevation of Mn-SOD activity. This observed increase in Mn-SOD activity in response to thioacetamide administration may be an attempt to detoxify the increased ROS and thus protect the tissue by dismutation of the $\text{O}_2^-$ radicals. Mn-SOD is well known for its role in the primary cellular defense by protecting the cell from deleterious effects of oxygen free radicals (Fridovich, 1986). Overexpression of SOD results in the enhanced production of hydrogen peroxide that could result in lipid peroxidation especially if it is not reduced by peroxidases (Ceballos-picot et al., 1991). A similar situation exists in the present study with enhanced SOD activity and decreased peroxidase activity levels in response to thioacetamide administration. Although an increase in SOD activity has a protective role, its increase with a simultaneous reduction in the activity of GPx is lethal to the tissue due to the accumulation of more hydroperoxides (Avraham et al., 1998; Devi et al., 1996). Similar observation of decreased GPx and GR activities was reported by Kosenko et al., (2003) in
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hyperammonemic states. The levels of total thiols indicate the redox state of the cell. The oxidation of thiols to disulfide (-S-S-), is a well-known sensitive indicator of oxidative stress (Sen et al., 2000). GR is a flavoprotein that catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) to glutathione (GSH) and maintains a balance of the reduced glutathione levels. Decreased GR activity observed in the present study indicates the impaired redox cycling of GSH leading to oxidative stress in the brains of FHF animals.

Decreased enzymatic activities of glutathione peroxidase and glutathione reductase, and an elevated Mn-SOD activity observed in the present study might lead to elevated levels of \( \text{H}_2\text{O}_2 \) (Devi et al., 1996; Dogru-Abbasoglu et al., 2001). This could lead to oxidative stress in the mitochondria of FHF rats. Similar observations have been made by Dogru-Abbasoglu and his group (Dogru-Abbasoglu et al., 2001) in the liver during thioacetamide induced hepatic failure. Oxidative stress has been shown to cause mitochondrial dysfunction (Paradies et al., 2001) which is implicated in many neurological disorders (Heales et al., 1999). This aspect, however, has to be further investigated.

TAA treatment of rats is characterized by marked elevation in serum and brain ammonia levels. The hyperammonemic state prevailing during TAA treatment may by itself lead to impairment of antioxidant enzyme functions as shown under in vivo conditions by the administration of ammonium acetate (Kosenko et al., 1997b;
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1999; 2003). Consistant with this Murthy et al (Murthy et al., 2000) have shown the suppression of ammonia induced swelling in \textit{in vitro} exposure of cells to antioxidant enzymes. Further studies of Staub \textit{et al}., (1994) and Norenberg \textit{et al}., (1991) also have shown that the free radicals may contribute to the cell swelling. However, the possible contribution of other factors that may be altered during TAA induced liver failure cannot be ruled out.

Glutathione is an abundant low molecular weight intracellular tripeptide, which is present in most of the cells. The presence of glutathione is required to maintain the normal function of the immune system. Glutathione is homeostatically controlled, both inside the cell and outside. Also glutathione is an important parameter that is implicated in the oxidative stress status of the cell. Furthermore, the cells produce many oxygen free radicals as a result of their normal functioning, resulting in a need for higher concentrations of antioxidants. Glutathione plays a crucial role in fulfilling this requirement. Glutathione also acts as a regulator for other antioxidants like vitamin C and E. It acts as a scavenger of reactive oxygen species, reactive nitrogen species, and potentially toxic oxidation products. It is also a substrate for the detoxifying enzymes like glutathione peroxidase, glutathione S-transferases and glutathione reductase. Glutathione exists in two forms, reduced (GSH) and oxidized (GSSG). The increase in the reduced form of glutathione in the nonsynaptic mitochondria of the thioacetamide treated rats may be due to the
increased activity of the γ-glutamyl-cysteine synthetase. Similar results were reported by Murthy et al., (2000) in astrocyte cultures exposed to hyperammonemia. Generally a cell tries to counteract the imposed oxidative stress by stimulating the synthesis of reduced glutathione. The increase in reduced GSH observed in the present study may be an effort made by the cell to maintain the reduced state of the cell. Along with the increase in the total GSH content the oxidized form of glutathione (GSSG) was also enhanced. This increase in the GSSG makes the cell to accumulate more of the oxidized form of glutathione. Although an increase in both GSH and GSSG were observed, the magnitude of increase of the GSSG was much higher than the increase in GSH. This is further evident from the ratio of GSH/GSSG which is an indicator of oxidative stress. The decreased GSH/GSSG ratio in the present study indicates that the tissue is being pushed to a state where the oxidized form of glutathione gets accumulated. This decrease in the ratio of GSH/GSSG along with the increased lipid peroxidation and decreased antioxidant defenses suggest the possible induction of oxidative stress in the nonsynaptic mitochondria of the TAA-induced FHF in rats.

The foregoing studies thus indicate that the FHF induced in rats by thioacetamide administration results in impaired structural and functional organization of nonsynaptic mitochondria, mainly by the increased lipid peroxidation and impaired antioxidant defenses. This induced oxidative stress in nonsynaptic mitochondria might be due to
impaired energy metabolism as a result of diversion of TCA cycle intermediates and ATP towards the detoxification of accumulated ammonia in brain tissue of rats during FHF. The impaired structure and function of nonsynaptic mitochondria may account for the neuronal death associated with FHF condition. Further studies, however, are required to study the impaired function of brain in these conditions.