CHAPTER V

EFFECT OF METHYL MERCURY TOXICITY ON BILIRUBIN, CREATININE AND ALT
Introduction

The accumulation of metal in our body occurs in the following order Hg > Cd > Zn in liver and Cd > Hg > Zn in kidney (Tandon, 2001). The cells have developed various strategies to cope with toxic stress. They include chemical modification of toxin, excretion by active transport to minimize concentration and to modify or strengthen cellular proteins to overcome the toxic stress.

Bilirubin is a conjugated tetrapyrroledicarboxylic acid, and is the principle pigment in bile. Bilirubin is the end product of heame catabolism in mammals, and most of the circulating bilirubin derived from senescent erythrocytes. At the end of the normal life span of erythrocytes, the heame dissociates from hemoglobin and is oxidised by the membrane-bound enzyme heame oxygenase (EC 1.14.99.3) to biliverdin, producing carbon monoxide as a by-product. Subsequent metabolism of biliverdin by the cytosolic enzyme biliverdin reductase (EC 1.3.1.24) gives rise to Bilirubin. Bilirubin exists in serum in four forms as unconjugated bilirubin, monoglucoronide, diglucoronide and albumin bound bilirubin. The last one is the most abundant form of bilirubin.

For many years bilirubin was considered only as a waste end product of heame catabolism which was either considered useless or toxic. During last few years, a number of intriguing biochemical properties of bilirubin have been discovered including
its particular role as an antioxidant (Ross McGeary, 2003).

Chinese traditionally use ox-gallstones in medicine which largely consist of calcium bilirubinate. In 1980s Stocker et al. found that bilirubin at micromolar concentration efficiently scavenged peroxyradicals either in homogenous solution or in multilamellar liposomes to a greater extent than alpha tocopherol. Further, Stocker and Ames 1987 showed that water-soluble bilirubin taurine conjugate could prevent radical induced oxidation of phosphatidyl choline in either micellar or multilamellar liposomes and that same conjugate greatly accelerate Cu^{2+} catalysed decomposition of linolic acid and hydroperoxide. They also showed that albumin bound bilirubin at concentration comparable to those present in normal plasma also had antioxidant activity and was capable of protecting albumin bound linoleic acid against radical induced oxidation. In competition studies albumin conjugated bilirubin was also found to out compete an equimolar concentration of uric acid for peroxyl radicals, but was less efficient in scavenging these radicals than was ascorbic acid (Stocker and Ernst, 1989). In later works Stocker and Ernst demonstrated synergistic interaction between bilirubin and vitamin E in inhibiting the oxidation of phosphotidyl choline liposomes. 

Low micromolar concentration of bilirubin was able to inhibit oxidation of these liposomes in a concentration dependent manner.
unlike ascorbic acid and glutathione which were ineffective. Frei and co-workers (1988) found bilirubin was more effective in protecting lipids from peroxidative damage than other endogenous antioxidants. Bilirubin scavanges superoxide radicals and protects serum albumin against auto-oxidation by OH radicals. It also acts as an antioxidant of peroxinitrite mediated protein oxidation in human blood plasma. Bilirubin has antioxidant effect similar to porphyrins. Asad et. al., 2000 have shown bilirubin inhibits L-dopa-Cu^{++} mediated DNA cleavage, and that bilirubin directly quenches OH radicals generated by L-dopa-Cu^{++} system. Dore et. al.1999 have shown bilirubin conjugates to human serum albumin are neuroprotective reversing the neurotoxic effects of H_{2}O_{2} on neuronal hippocampal cultures at concentrations as low as 10 nmoles.

The enzyme Alanine aminotransferase (ALT) previously known as Serum Glutamic Pyruvate Transaminase (SGPT) is a cytoplasmic enzyme that catalyses the transamination of \( x \) ketoglutarate and L-alanine, forming glutamate and pyruvate. This chemical reaction is irreversible. This enzyme is also called as Alanine transaminase. The highest activity of ALT is found in hepatocytes and striated muscles (skeletal and cardiac muscles) therefore, increased serum ALT accompanies hepatocellular injury or necrosis of striated muscles ( Bain, 2003 ). This enzyme is
primarily found in liver but to a lesser degree in the heart and other tissues, with cell injury or death ALT escapes from cytosol. It is released from the liver cells into blood stream often before jaundice appears resulting in abnormally high serum levels.

Determination of ALT activity is a relatively sensitive indicator of hepatic damage in certain animal species and can help to determine whether further diagnostic test i.e., creatine kinase, bile acid concentration or liver biopsy are necessary. Mechanism of increased activity of ALT in serum include enzyme released from damaged cells or induction of enzyme activity (increased enzyme synthesis) from drug administration. Release of ALT from cytosol can occur secondary to cellular necrosis or as a result of cellular injury with membrane damage and bleb formation. Very high ALT levels (50 times normal) suggest viral or severe drug induced hepatitis or other hepatic diseases with extensive necrosis (death of liver cells). Moderate to high-level increase in enzyme activity may indicate infectious mononucleosis, chronic hepatitis, intrahepatic cholestasis or cholecystitis, early or improving viral hepatitis or severe hepatic congestion due to heart failure. Marginal elevations occasionally occur in acute myocardial infarction, secondary hepatic congestion. Many medications produce hepatic injury by competitively interfering with cellular metabolism. Falsely elevated ALT levels can follow use of barbiturates, narcotics, methotrexate
etc. In dogs, cats, rabbits and primates ALT activity is highest in hepatocytes. Therefore, serum ALT is specific for liver disease however; the measurement of serum ALT does not test hepatic integrity alone because high ALT activity may occur with striated muscle necrosis or injury (Valentine, 1990). Ruminants, pigs, horses and birds have a much lower level of hepatocellular ALT activity. In these species increase of ALT is usually a reflection of skeletal muscle necrosis.

Like many other organic bases, creatinine is filtered at glomerulus and secreted into tubular lumen by proximal tubular epithelial cells. Creatinine is an organic product of muscle protein metabolism. Its level is a reflection of bodies' muscle mass. Low levels are sometimes seen in kidney damage, protein starvation, liver disease or pregnancy. Elevated levels are sometimes seen in kidney disease due to the kidneys job of excreting creatinine, muscle degeneration, and some drugs involved in impairment of kidney function. Phosphogen is a group of compounds that acts as a storage form of high-energy phosphate. These include creatine-phosphate occurring in vertebrate skeletal muscle, heart, spermatozoa and brain. Arginine phosphate occurs in invertebrate muscle. Under physiologic concentration phosphogens permit ATP concentration to be maintained in muscles when ATP is rapidly being utilized as a source of energy for muscle contraction. On one
hand, when ATP/ADP ratio is high, their concentration can build up to act as a store for high-energy phosphate. In muscle creatine phosphate shuttle has been described that transports high-energy phosphate from mitochondria to sarcolemma and acts as a high-energy phosphate buffer. Creatine is synthesized from glycine and arginine precursors in muscle from creatine phosphate by irreversible non-enzymatic reaction. Creatine-phosphate is unstable and undergoes slow, spontaneous degradation to Pi and creatinine, which is excreted from muscle cell to plasma to urine. Both creatine and its high-energy form phosphocreatine are present in muscle, brain and blood. The 24-hour excretion of creatinine in urine of a subject is remarkably constant from day to day proportionate to muscle mass.

This experiment was designed to study the effect of MeHg on liver and kidney with a view to assess the antioxidant potential of bilirubin and in vivo, its relation with neurobehavioural parameters if any. Tests were also carried out to study the effect of alpha-lipoic acid on listed parameters.

**Protocol**

For various sets of biochemical studies different groups comprising six animals each were used. Animals from Group I served as control while, animals of Group II, III and IV were used as experimental sets. Group II animals were given Methyl mercury
Chloride (MeHgCl) 1 mg/kg body weight. Animals of Group III received Alpha Lipoic Acid 35 mg/kg body weight. Group IV animals were given MeHgCl 1 mg/kg body weight and Alpha Lipoic Acid 35-mg/kg body weight. All groups were treated once a day each for seven days intraperitonially. On eighth day the animals were tested for Photoactometer, Y-maze and Rota rod tests. Blood samples collected were later processed for the assay of bilirubin, creatinine and ALT as described in detail in Materials and Methods.

**Result**

Methyl mercury intoxicated rats showed decrease in antioxidant bilirubin, (Table 24) but when these animals were treated with alpha lipoic acid (ALA) significant improvement in bilirubin content was noticed. The rats with improved bilirubin were given ALA along with methyl mercury. These rats demonstrated better results on conducted tests of motor function and memory viz., rotarod and Y-maze tests. The effect of methyl mercury on motor activity is shown in Table 26. 200% motor activity decline was observed in methyl mercury intoxicated rats as compared to control. Alpha lipoic acid offered protection against methyl mercury induced decline in motor activity by 50% as compared to control. The effect of methyl mercury on memory as tested by Y-maze test is shown in Table 25. Methyl mercury fed
TABLE 24.

Alteration of serum bilirubin mg/dl following methyl mercury (1mg/kg b. wt) and α-lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MeHg</th>
<th>MeHg+ALA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.65 ± 0.42</td>
<td>0.38 ±0.03**</td>
<td>0.45 ± 0.034*</td>
<td>0.49 ± 0.032*</td>
</tr>
<tr>
<td></td>
<td>(41.53%)</td>
<td>(30.76%)</td>
<td>(24.61%)</td>
<td></td>
</tr>
</tbody>
</table>

Values given are mean of ± S.E of six animals.
Figures in parenthesis indicate change compare to control

*p < 0.05*  
**p < 0.01
Alteration of serum bilirubin mg/dl following methyl mercury (1mg/kg b. wt) and alpha lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.
TABLE 25.

Effect of methyl mercury (1mg/kg body wt) and alpha lipoic acid (35 mg/kg body wt) intraperitoneally on albino rats for seven days as assessed by Y-maze test.

<table>
<thead>
<tr>
<th>Test group</th>
<th>Alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.83 ± 0.16</td>
</tr>
<tr>
<td>Methyl mercury</td>
<td>1.16 ± 0.33*</td>
</tr>
<tr>
<td></td>
<td>(75.98%)</td>
</tr>
<tr>
<td>Methyl mercury +</td>
<td>3.46 ± 0.3*</td>
</tr>
<tr>
<td>Alpha lipoic acid</td>
<td>(28.36%)</td>
</tr>
</tbody>
</table>

Values expressed are alterations per 8 minutes by a rat. Values given are mean of ± S.E of six animals.
Figures in parentheses indicate change compared to control.

*p < 0.05.
Effect of methyl mercury (1mg/kg body wt) and alpha lipoic acid (35 mg/kg body wt) intraperitoneally on albino rats for seven days as assessed by Y-maze test.
TABLE 26.

Effect of methyl mercury (1mg/kg body wt) and alpha lipoic acid (35 mg/kg body wt) intraperitoneally on albino rats for seven days as assessed by Rota rod test.

<table>
<thead>
<tr>
<th>Test group</th>
<th>% error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2</td>
</tr>
<tr>
<td>Methyl mercury</td>
<td>6*</td>
</tr>
<tr>
<td></td>
<td>(-200%)</td>
</tr>
<tr>
<td>Methyl mercury + Alpha lipoic acid</td>
<td>1*</td>
</tr>
<tr>
<td></td>
<td>(+50%)</td>
</tr>
</tbody>
</table>

Values expressed are % error (fall) by rats on Rota rod at 10 rpm before completion of 300 seconds. Values given are mean of ± S.E of six animals. Figures in parentheses indicate change compared to control.

*p < 0.05.
Effect of methyl mercury (1mg/kg body wt) and alpha lipoic acid (35 mg/kg body wt) intraperitoneally on albino rats for seven days as assessed by Rota rod test.
TABLE 27.

Alteration of serum creatinine (mg/dl) following methyl mercury (1mg/kg b. wt) and α-lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MeHg</th>
<th>MeHg + ALA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.54 ± 0.119</td>
<td>0.95 ± 0.041*</td>
<td>0.91 ± 0.052*</td>
<td>0.87 ± 0.055*</td>
</tr>
</tbody>
</table>

Values given are mean of ± S.E of six animals.

*p < 0.05.
Alteration of serum creatinine (mg/dl) following methyl mercury (1mg/kg b. wt) and alpha lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.
TABLE 28.

Alteration of alanine aminotransferase (ALT) following methyl mercury (1mg/kg b. wt) and α-lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MeHg</th>
<th>MeHg+ALA</th>
<th>ALA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>111.83 ± 4.86</td>
<td>141.5 ± 5.8*</td>
<td>131± 2.8**</td>
<td>129.8 ± 2.02**</td>
</tr>
</tbody>
</table>

Values given are mean of ± S.E of six animals.

*p < 0.05.  **p < 0.01
Alteration of alanine aminotransferase (ALT) following methyl mercury (1mg/kg b. wt) and alpha lipoic acid (35mg/kg b.wt) intoxication i.p. individually as well as in combination for seven days.
rats demonstrated a decline of 75.98% memory compared to control. But when mercury intoxicated rats were given alpha lipoic acid significant improvement in memory was noted at 28.36% in comparison to control (p < 0.05).

Methyl mercury dozed experimental animals exhibited a low level of serum creatinine (Table 27) and enzyme alanine aminotransferase (ALT) (Table 28) in comparison to controls. This indicated a poor state of hepatic and renal system. When the rats were given methyl mercury along with ALA were subsequently tested on given parameters they showed improvement in levels of serum creatinine and ALT. They also demonstrated improved behavioural indices of memory and motor function suggesting restoration of damage activity.

Discussion

Hyperbilirubinemia is commonly observed in newborn humans, and possible protective role of bilirubin in neonates has long been debated. Evidence has been found for the protective effect of bilirubin to neonatal rats exposed to hyperoxia against serum oxidative damage in first few days of life (Dennery, 1995). The new concept advocates bilirubin as a biological endogenous antioxidant. Some research groups argue it to be a more potent antioxidant than glutathione (Baranano, 2002). At physiologically
relevant concentrations bilirubin is useful in protecting the body against oxidative damage.

In the current study it was found that decreased level of bilirubin on exposure to methyl mercury correlated well with neurobehavioural indices. Improved bilirubin concentrations corresponded with enhanced motor and memory functions in tested rats when rats were exposed to methyl mercury along with alpha lipoic acid. There are new studies devoted to assess the antioxidant status of bilirubin and its role in neuroprotection. The results of present study agree well with that of others. Serum bilirubin concentration decreases in patients with long duration of amyotropic lateral sclerosis (Ilzecka & Stelmsiak, 2003). They also found improved bilirubin levels associated with improved state of patient and concluded that a decrease in bilirubin concentration might diminish its protective effect against oxidative injury and could accelerate motor neuron degeneration. The major plasma antioxidants - bilirubin, uric acid and albumin were found to be significantly low in first episode of schizophrenia (Reddy, 2003). The total and individual antioxidants decreased in plasma of chronic schizophrenia patients. In another study conducted by Liu, 2003 it was found that bilirubin prevented autoimmune encephalomyelitis (EAE) in experimental animals. It also suppressed clinical EAE. It is concluded that neuroprotective
effects of bilirubin are observed because it prevents blood-brain barrier from free radical induced permeability changes. Asad et. al. 2000 have shown bilirubin inhibits L-dopa-Cu\textsuperscript{2+} mediated DNA cleavage and that bilirubin directly quenches OH radicals generated by L-dopa-Cu\textsuperscript{2+} system. Dore et. al. 1999 have shown bilirubin conjugates to human serum albumin are neuroprotective reversing the neurotoxic effects of \(H_2O_2\) on neuronal hippocampal cultures at concentrations as low as 10 nmoles. As little as 10 nmoles bilirubin provides cytoprotection against 10,000 fold high concentration of \(H_2O_2\). This is possible by bilirubin redox system; during conversion of biliveridin to bilirubin the high amount of quenching of free radicals takes place. Bilirubin is a lipophilic compound associated with cell membranes and protects them against free radical injury.

The ALT and creatinine values demonstrated enhancement on exposure to methyl mercury in our study. Mercuric intoxication produced alteration in ALT & AST of \textit{Notopterus notopterus} (Verma, 1984). 10 ppm methyl mercury significantly altered ALT leakage after 60 min. on isolated rat hepatocytes. El Demerdash, 2001 also found rise in ALT values on exposure to HgCl\textsubscript{2}. Serum creatinine, urea increased in rats exposed to mercuric chloride (Rumbeiha, 2000). Renal dysfunction caused by exposure to 5ppm mercury increased
plasma creatinine level (Yasutake, et. al., 1997). The rats are
maintained on high protein diet exhibited normal serum Cr and
bilirubin with in 4 days following Hg exposure. Our results
demonstrate that when Alpha Lipoic acid was given along with
methyl mercury it helped to bring back the creatinine and enzyme
ALT level restoring the hepatocellular damage. ALA improved
protein status in methyl mercury exposed rats as discussed earlier
in chapter III. High dietary protein regimens may protect from
mercury nephrotoxicity by reducing mercury uptake to second
segment of proximal tubules during initial period of exposure to
Moreover, there are evidences from previous work that ALA
restores liver function. It helps in restoring liver function in cases
of alcohol, mushroom, carbon tetrachloride poisoning and
incidences of metal intoxication, i.e any where when oxidative
stress is involved (Bustamante, et. al., 1998). According to
Lynch, 2001 lipoic acid confers protection against oxidative injury
in neuronal and non-neuronal tissue.