CHAPTER- 5
CHAPTER –5

EVALUATION OF MEMBRANE BOUND AND MITOCHONDRIAL ENZYMES IN CONTROL AND EXPERIMENTAL ANIMALS

5.1. INTRODUCTION

Mitochondria are an important intracellular source and target of reactive oxygen species. Mitochondrial aging is characterized by destruction of structural integrity of membranes, leading to a decline in mitochondrial membrane fluidity and activities of enzymes associated with membrane lipids (Arivazhagan et al., 2001). As the activities of most enzymes are regulated by the physicochemical state of the lipid environment of the membrane, it seems likely that impaired mitochondrial membrane function brought about by aging could be related to free radical reactions such as lipid peroxidation, ROS and their metabolites generated by components of electron transport chain during mitochondrial respiration. This potential self-destruction renders the mitochondrial membrane more vulnerable to oxidative damage than other cellular membranes (Chen and Yu, 1994).

Peroxidation of membrane is accompanied by alteration of structural and functional characteristics of membranes. Lipid peroxidation changes the activities of various enzymes. ATPases are very sensitive to peroxidation reactions and abnormal lipoperoxides affect ATPase activities. ATPases are intimately associated with the plasma membrane and participates in the energy requiring translocation of sodium, potassium, calcium and magnesium ions (Senthilnathan, et al., 2006).

\[ \text{Na}^+ / \text{K}^+ - \text{ATPase (also known as the Na}^+ / \text{K}^+ \text{ pump) sodium-potassium pump, or simply sodium pump is an enzyme located in the plasma membrane (to be specific, an electrogenic transmembrane ATPase) in all animals. Active transport is responsible for cells containing relatively high concentrations of potassium ions but low concentrations of sodium ions. The mechanism responsible for this is the sodium-potassium pump, which moves these two ions in opposite directions across the plasma membrane. This was investigated by following the passage of radioactively labeled ions across the plasma membrane of certain cells. It was found that the concentrations of sodium and} \]

\[ \text{...} \]
potassium ions on the two other sides of the membrane are interdependent, suggesting that the same carrier transports both ions. It is now known that the carrier is an ATP-ase and that it pumps three sodium ions out of the cell for every two potassium ions pumped.

Mg$^{2+}$ is a cofactor of more than three hundreds of enzymatic reactions and is especially important for those enzymes that use nucleotides as cofactors or substrates. This is true for phosphotransferases and hydrolases such as ATPases which are of the central importance in the biochemistry of the cell, particularly in energy metabolism. In addition, Mg$^{2+}$ was required for protein and nucleic acid synthesis, the cell cycle cytoskeleton and mitochondrial integrity and for the binding of substrates to the plasma membrane. Mg$^{2+}$ frequently modulates ion transport by pumps, carriers and channels, and thereby, may modulate signal transduction and the cytosolic concentration of electrolytes, such as Ca$^{2+}$ and K$^+$ (Smith and Maguire, 1993). Deregulation of intracellular Ca$^{2+}$ homeostasis the unbalance between pro-oxidant reactions and antioxidant defenses. This modification of cell redox state is important events involved in hypertrophied and failing myocardium. Aim of the present investigation is to evaluate the mitochondrial and membrane bound enzymes in experimental animals.

5.2. MATERIALS AND METHODS

5.2.1. Mitochondria isolation

The liver was quickly removed, chopped into small pieces and placed in ice-cold isolation buffer for mitochondria (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.5 mM EDTA and 0.5% bovine serum albumin). After being homogenized, the homogenate was centrifuged at 750 g for 10 min and supernatant was collected and then it was centrifuged at 10,000 g for 10 min. The mitochondrial pellet was washed twice with isolation buffer, and then resuspended in the same buffer solution. The freshly prepared mitochondria were used to determine the antioxidant activities and TCA cycle enzyme.

5.2.2. Assay of Na$^+$ K$^+$ ATPase (Bonting, 1970)

1.0 ml of Tris buffer and 0.2 ml of each of the above reagents were mixed, together. Thus the assay medium in a final volume of 2.0 ml, contained 92mM tris buffer, 50mM MgSO$_4$, 60mM NaCl, 1mM EDTA and 4mM ATP. After 10 minutes,
equilibrium at 37°C in an incubator, reaction was started by the addition of 0.1 ml of homogenate. The assay medium was incubated for 15 minutes. After incubation, the reaction was arrested by the addition of 1.0 ml of 10% TCA. The enzyme activity is expressed as micromoles of Pi liberated/min/mg protein.

5.2.3. Assay of Mg$^{2+}$ ATPase (Ohnishi et al., 1982)

The assay was initiated by the addition of 0.1 ml of homogenate to an incubation medium containing 0.1 ml of water and 0.1 ml of each of the above reagents. The final concentration of tris buffer, MgCl$_2$ and ATP were 75mM, 5mM and 2mM respectively with total incubation volume of 0.5 ml. The reaction was terminated after 15 minutes by the addition of 1.0 ml of 10% TCA. The enzyme activity was expressed as µ moles of Pi liberated/min/mg protein.

5.2.4. Assay of Ca$^{2+}$ ATPase (Ohnishi et al., 1982)

The assay was estimated by the addition of 0.1ml of homogenate to an incubation medium containing 0.1ml of water and 0.1ml of each of the above reagents. The final concentration of Tris buffer, CaCl$_2$ and ATP were 75mM, 5mM, 2mM with total incubation volume of 0.5ml. The reaction was terminated after 15 minutes by the addition of 1.0ml of 10% TCA. The enzyme activity was expressed as micromoles of Pi liberated / min / mg protein.

5.2.5. Estimation of phosphorus (Fiske and Subbarow, 1925)

Into a series of test tubes pipette out 1.0–5.0 ml of working standard solution corresponding to µg values 8P40. 1.0 ml of the sample solution was taken in separate test tubes. The volume in all the tubes was made up to 8.6 ml with distilled water. Set up a blank with 8.6 ml of distilled water. Added 1.0 ml of 2.5% ammonium molybdate and 0.4 ml of ANSA to all the tubes. Mixed well and allowed to stand for 10 minutes. The blue color developed was read at 660 nm in a spectrophotometer.

5.2.6. Assay of isocitrate dehydrogenase (IDH) (Slater and Bonner, 1952)

0.3 ml of buffer solution was taken in a test tube and 0.2 ml of substrate, 0.3ml of manganese chloride and 0.2 ml of the mitochondrial suspension were added. A control tube was also prepared simultaneously. 0.2 ml of co-enzyme solution was added to the test and 0.2 ml of saline was added to control tubes. After mixing well, both the tubes were incubated for 60 mins. 1 ml of colour reagent (DNPH) was added
to both the tubes followed by 0.5 ml of EDTA. The tubes were kept at room temperature for 20 mins and 10 ml of 0.4 N NaOH was added to the tubes. A blank was run simultaneously. The colour was measured at 390 nm. A standard curve was prepared using α – ketogulutarate. The activity of isocitrate dehydrogenase is expressed as nanomoles of α – ketogulutarate liberated / min / mg of total protein under incubation curve.

5.2.7. Assay of α-Keto dehydrogenase

α KDH activity in mouse mitochondria was measured fluorimetrically. The reaction medium was composed of 50 mM KCl, 10 mM HEPES, pH 7.4, 20 µg/ml alamethicin, 0.3 mM thiamine pyrophosphate (TPP), 10 µM CaCl₂, 0.2 mM MgCl₂, 5 mM α-ketoglutarate, 1 µM rotenone, and 0.2 mM NAD⁺. The reaction was started by adding 0.14 mM CoASH to permeabilized mitochondria (0.1– 0.25 mg/ml). Reduction of NAD⁺ was followed at 460 nm emission after excitation at 346 nm.

5.2.8. Assay of Succinate Dehydrogenase (SDH) (Slater and Bonner, 1952)

Added 1.0 ml of phosphate buffer, 0.1 ml of EDTA, 1.0 ml of KCN was added and made up to 2.9 ml with water. Note the extinction at 455 nm, then started the reaction by the addition of enzyme and followed the change in extinction during the first two min. Initial rates were taken as a measure of activity. A blank rate (all reagents except succinate) must be determined separately. In this determination, 1 mole of succinate reduces 2 moles of potassium ferricyanide. Concentration of potassium ferricyanide rates can be measured by following the reaction at 420 nm (ε = 1.03 x 10⁵ cm). The enzyme activity is expressed as micromoles of succinate produced/min/mg protein under incubation condition.

5.2.9. Assay of Malate Dehydrogenase (MDH) (Mehler et al., 1948)

The reaction mixture contained the following reagents and enzyme in a total volume of 3.0 ml. 75 µM of phosphate buffer, 0.15 µM of NADH and 0.76 µM of oxaloacetate. The reaction was carried at 25°C and was started by the reagents by the addition of enzyme preparation. The control tubes contained all reagents except NADH. The change in OD at 340 nm was measured for 2 min at the interval of 15 secs.

The activity of the enzyme was expressed as micromoles of NADH oxidized/min/mg protein using the extinction coefficient of NADH as 6.22 x 10³.
5.3. RESULTS

Table 5.1. represents the membrane bound enzymes ATPases such as Sodium-Potassium (Na\textsuperscript{+}K\textsuperscript{+}) ATPase, Magnesium (Mg\textsuperscript{2+}) ATPase, Calcium (Ca\textsuperscript{2+}) ATPase in control and experimental animals. The activity of Ca\textsuperscript{2+} ATPase enzymes found to be increased significantly in CCl\textsubscript{4} induced compared to control group of mice. There was a decrease in the activities of Ca\textsuperscript{2+} ATPases observed in experimental mice treated with C. asiaticum, lycorine and silymarin as compared with CCl\textsubscript{4} administered mice. The activity of Mg\textsuperscript{2+} ATPase enzymes increased significantly in CCl\textsubscript{4} induced compared to control group of mice. There was a decrease in the activities of Mg\textsuperscript{2+} ATPases observed in experimental mice treated with C. asiaticum, lycorine and silymarin as compared with CCl\textsubscript{4} administered mice. The activity of Na\textsuperscript{+} K\textsuperscript{+} ATPase decreased significantly (P<0.05) in the liver of CCl\textsubscript{4} induced mice compared with control mice. However, treatment with C. asiaticum and lycorine to CCl\textsubscript{4} induced mice increased significantly (P<0.05) the activity of Na\textsuperscript{+} K\textsuperscript{+} ATPase in the liver compared to CCl\textsubscript{4} induced. Silymarin treated mice also showed a similar trend as C. asiaticum and lycorine. No significant statistical changes were distinguished in mice treated with C. asiaticum and lycorine alone compared to that of control group of mice.

Table 5.1. Effect of Crinum asiaticum and lycorine on Na\textsuperscript{+} K,\textsuperscript{+} ATPases and Mg\textsuperscript{2+} Ca\textsuperscript{2+} ATPases.

<table>
<thead>
<tr>
<th>Groups</th>
<th>*Na\textsuperscript{+}K\textsuperscript{+}ATPases</th>
<th>*Ca\textsuperscript{2+}ATPase</th>
<th>*Mg\textsuperscript{2+}ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.25 ± 0.04</td>
<td>1.32 ± 0.07</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td>C. asiaticum alone</td>
<td>1.23 ± 0.01</td>
<td>1.37 ± 0.02</td>
<td>0.76 ± 0.02</td>
</tr>
<tr>
<td>Lycorine alone</td>
<td>1.26 ± 0.02</td>
<td>1.36 ± 0.05</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>CCl\textsubscript{4} alone</td>
<td>0.52 ± 0.02\textsuperscript{a}</td>
<td>2.05 ± 0.01\textsuperscript{a}</td>
<td>1.14 ± 0.07\textsuperscript{a}</td>
</tr>
<tr>
<td>C. asiaticum + ccl\textsubscript{4}</td>
<td>0.85 ± 0.01\textsuperscript{b}</td>
<td>1.67 ± 0.06\textsuperscript{b}</td>
<td>1.03 ± 0.01\textsuperscript{b}</td>
</tr>
<tr>
<td>Lycorine +ccl\textsubscript{4}</td>
<td>0.64 ± 0.07\textsuperscript{b}</td>
<td>1.64 ± 0.05\textsuperscript{b}</td>
<td>0.92 ± 0.06\textsuperscript{b}</td>
</tr>
<tr>
<td>Silymarin+ ccl\textsubscript{4}</td>
<td>1.18 ± 0.01\textsuperscript{b}</td>
<td>1.52 ± 0.03\textsuperscript{b}</td>
<td>0.78 ± 0.04\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\*mM of inorganic phosphorous/ mg protein. Values of results are expressed as Mean±.SD for six mice: \textsuperscript{a}P<0.05 compared with control group of mice. \textsuperscript{b}P<0.05 compared with CCl\textsubscript{4} induced group of mice

Table 5.2 represents the restorative effect of C. asiaticum, lycorine and silymarin on CCl\textsubscript{4} induced mitochondrial tricarboxylic acid cycle enzyme (TCA) alterations in control and experimental animals. The activity of isocitrate dehydrogenase (ICDH) reduced in the CCl\textsubscript{4} administered group of mice. However, treatment with C. asiaticum and lycorine significantly increased the activity of...
isocitrate dehydrogenase compared with CCl₄ induced group of mice. Silymarin treated mice showed significant increases of isocitrate dehydrogenase activity compared with CCl₄ induced group of mice. The present results indicate that the C. asiaticum and lycorine protect the mitochondrial damage as similar to silymarin. The activity of alpha keto glutarate (KDH) dehydrogenase diminished in the CCl₄ induced mice. Where as after treatment with C. asiaticum and lycorine showed a significant increase in the activity of alpha ketoglutarate dehydrogenase (P≤0.05) compared to the CCl₄ induced mice. Similarly, silymarin treatment to CCl₄ induced mice exhibit the increased the activity of alpha keto glutarate dehydrogenase. The activity of succinate dehydrogenase (SDH) decreased in the CCl₄ induced mice compared with control group of mice. However, C. asiaticum, lycorine and silymarin therapy reversed the altered activity of Succinate dehydrogenase when compared (P≤0.05) with CCl₄ induced group of mice. The activity of malate dehydrogenase (MDH) significantly reduced in CCl₄ induced group of mice. But after C. asiaticum and lycorine treatment the activity of malate dehydrogenase increased compared with CCl₄ induced group of mice. Similarly, silymarin treatment to CCl₄ induced mice showed significant increases of malate dehydrogenase compares to CCl₄ induced mice. There was no significant deviation changes observed in mice treated with C. asiaticum and lycorine alone compared to that of control group of mice.

Table 5.2. Effect of Crinum asiaticum and lycorine on mitochondrial TCA enzymes

<table>
<thead>
<tr>
<th>Groups</th>
<th>*ICDH nmol/min/mg protein</th>
<th>**KDH nmol/min/mg protein</th>
<th>***SDH µmol/min/mg protein</th>
<th>****MDH µmol/min/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>835.23 ± 23.5</td>
<td>184.2 ± 12.5</td>
<td>46.26 ± 3.0</td>
<td>356.02 ± 5.6</td>
</tr>
<tr>
<td>C. asiaticum alone</td>
<td>833.15 ± 20.2</td>
<td>186.5 ± 12.8</td>
<td>47.35 ± 4.2</td>
<td>360.05 ± 6.3</td>
</tr>
<tr>
<td>Lycorine alone</td>
<td>838.53 ± 24.1</td>
<td>189.2 ± 14.5</td>
<td>47.63 ± 3.2</td>
<td>358.45 ± 7.2</td>
</tr>
<tr>
<td>CCl₄ alone</td>
<td>712.52 ± 32.5</td>
<td>096.2 ± 9.2</td>
<td>35.34 ± 1.3</td>
<td>298.32 ± 9.2</td>
</tr>
<tr>
<td>C. asiaticum + CCl₄</td>
<td>785.65 ± 25.1</td>
<td>135.1 ± 6.7</td>
<td>40.15 ± 1.4</td>
<td>313.71 ± 2.1</td>
</tr>
<tr>
<td>Lycorine + CCl₄</td>
<td>780.45 ± 34.5</td>
<td>126.6 ± 5.2</td>
<td>38.12 ± 6.4</td>
<td>307.96 ± 1.9</td>
</tr>
<tr>
<td>Silymarin + CCl₄</td>
<td>819.26 ± 14.3</td>
<td>168.2 ± 8.5</td>
<td>43.58 ± 6.1</td>
<td>342.71 ± 8.4</td>
</tr>
</tbody>
</table>

*nmol of a-ketoglutarate formed/min/mg protein, **nmol of ferrocyanide formed/min/mg protein, ***µmol of succinate oxidized/min/mg protein, ****µmol of NADH oxidized/min/mg protein. Values of results are expressed as Mean±SD for six mice. * P<0.05 compared with control group of mice. * P<0.05 compared with CCl₄ induced group of mice.
5.4. DISCUSSION

Carbon tetrachloride is one of the most favorite subjects used for creating liver injury in animal studies. Metabolic activation of short half-life reactive intermediate product was shown to be related with the hepatotoxic effects of CCl₄ (Halliwell and Gutteridge, 1984). Reductive dehalogenation, formation of covalent bonds with radicals, protein synthesis inhibition (mainly of apolipoproteins), lipid deposition, reduction of calcium sequestration, apoptosis, and fibrosis were main processes seen during CCl₄-induced liver injury (Boll et al., 2001). Carbon tetrachloride damages plasma and erythrocyte membranes (Lepage et al., 1988) CCl₄ also acts on hepatic ATP content (Mourelle and Franco, 1991). It was concluded that Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase activities were altered along with lipid composition of erythrocyte membranes after administration of CCl₄ for 7–8 weeks (Mourelle and Franco, 1991). Similarly, present results showed significant reduction in Ca²⁺/Mg²⁺-ATPase activities in the CCl₄ induced group of mice. However, after treatment with *C. asiaticum* and lycorine the altered activities were reversed. Hence, this result denotes that the membrane bound enzymes protective nature of *C. asiaticum* and lycorine in mice.

ATPases of the cardiac cells play a significant role in the contraction and relaxation cycles of the cardiac muscle by maintaining normal ion levels (Na⁺ and K⁺) within the myocytes. Changes in the properties of these ion pumps affect the cardiac function. Any disturbance or inactivation of these enzymes can alter the concentration of ions. Changes in ionic concentrations can bring about diverse types of cell injury and ultimate cell death (Trump et al., 1980). Na⁺/K⁺ pump is sensitive to changes in membrane phospholipids content because phospholipid molecules have a significant role in regulation of Na⁺/K⁺-ATPase activity. It was concluded that changes in phospholipid content of Na⁺/K⁺-ATPase subunits alters the activity of Na⁺/K⁺-ATPase activity (Roelofsen et al., 1981). The intermediate products of lipid peroxidation was shown to abolish the functions and structures of cell membrane by decreasing membrane viscosity, increasing membrane permeability, inactivation of membrane-dependent enzymes and loss of essential fatty acids (Van Ginkel and Sevanian, 1994). This condition changes the structural and functional integrity, but it also changes the activities of many membrane-bounded enzymes, which contain ATPase (Rauchova et
Na\textsuperscript{+}/K\textsuperscript{+}-ATPase protects intracellular increase of electrolytes by protecting Na\textsuperscript{+}-K\textsuperscript{+} ion equilibrium. In accordance with other studies, it was found that Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity decreased in CCl\textsubscript{4} treated group. Thus it was concluded that CCl\textsubscript{4} causes membrane injury by inducing lipid peroxidation and impairs the interaction between Na, K ions, and Na, K pump resulting with reduction of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity. However, mice administered with \textit{C. asiaticum} and lycorine significantly reversed to nearly normal level.

Mitochondria have been recognized as a major physiological sources of reactive oxygen species namely superoxide anions and hydroperoxides, which arise as consequences of oxygen reduction (Zhang \textit{et al.}, 1990). Phospholipids compressing the lipid bilayer of mitochondrial membranes are rich in poly unsaturated fatty acids, thus they are prone to free radical attack, resulting in their peroxidation decreased uptake of oxygen and production of ATP (Del Maestro, 1980).

During ischemia, pronounced enhancement of lipid peroxidation was seen in the mitochondria. The dehydrogenase of TCA cycle enzymes could have been affected by the free radicals on CCl\textsubscript{4}. Inhibition of the TCA cycle enzymes by reactive oxygen species (ROS) may affect the mitochondrial substrate oxidation, resulting in reduced oxidation of substrates, reduced rate of transfer of reducing equivalents to molecular oxygen and depletion of cellular energy (Capetenaki, 2008). Carbon tetrachloride significantly reduced ICH, KDH, SDH, and MDH in the mitochondria. However, treatment of \textit{C. asiaticum} and lycorine had shown a significant increase in the levels of ICDH, KDH, SDH, and MDH in the mitochondria.

The results of the present study indicate that administration of \textit{C. asiaticum} and lycorine was effective in improving mitochondrial TCA cycle activity and enhance the membrane bound ATPase in CCl\textsubscript{4} induced group of mice. It was concluded that \textit{C. asiaticum} and lycorine could be an effective therapeutic agent in treatment of free radical related disorders.