Chapter IV

EFFECT OF 8-ENDOTOXIN ON LIVER FUNCTIONS AND METABOLISMS
Introduction

Liver has a pivotal role in regulation of physiological process. It is involved in several vital functions such as metabolism, secretion and storage. Furthermore, liver is called the detoxification organ of mammals. Liver diseases are among the most serious ailment. They may be classified as acute or chronic hepatitis, hepatosis and cirrhosis. Liver diseases are mainly caused by chemicals but hepatotoxic effects of natural toxins is very common and studied extensively [226, 227]. An investigation on the hepatotoxic effects of δ-endotoxin in mice in certain aspects has been carried out.

Materials

δ-Endotoxin (Prepared in our laboratory), D-glucose (Glaxo Laboratories, India), Sodium pyruvate (Sigma, USA), 2,4-Dinitrophenyl hydrazine, L-aspartic acid, DL-alanine, α-oxoglutaric acid (E.Merck, Germany), Heparin (Biological Evans Ltd, India), Disodium orthoarsenate, Potassium hydroxide, Sodium carbonate, Sodium bicarbonate, Rochelle salt, anhydrous Sodium sulphate, Zinc sulphate, Copper sulphate, Dipotassium hydrogen Phosphate, Ammonium molybdate, Barium hydroxide, Potassium hydrogen Phosphate, Phenolphthalein (Glaxo Laboratories, India), Alcohol (Bengal Chemical, Calcutta), Hydrochloric acid, Sulphuric acid (International Chemical Industries, Calcutta), Sulphanilic acid, Sodium nitrite (Qualigens, India), Bilirubin, p-nitrophenyl phosphate, Hydroxy methyl amino methane (Tris), Alkaline phosphatase, Cholesterol (Sigma, USA), Chloroform, Solvent ether, Methanol, Glacial acetic acid, Formaldehyde, Benzene, Eosin and Hematoxyline (E. Merck, Germany).

Methods

Inbred swiss mice of either sex weighing between 20-25 g. were used. The mice were fed a standard pellet food (Hindusthan Lever, India) and given water *ad libitum*. 
The animals were divided into three groups — Group A: Saline control (5.0 ml physiological saline/kg body weight), Group B: δ-endotoxin at a dose level of 70 kitu/kg and Group C: δ-endotoxin at a dose level of 140 kitu/kg. All the treatments were carried out for six weeks, thrice in a week through intraperitoneal route.

Animals were handled gently and decapitated 18 hrs. after the last dose and pieces of liver tissue from each animal were removed for glycogen estimation. Blood of each animal was analyzed for estimation of glucose, serum aspartate aminotransferase (SGOT), serum alanine aminotransferase (SGPT), bilirubin (conjugated and total), alkaline phosphatase (ALP), fatty acids and cholesterol levels.

i) Estimation of liver glycogen [228]

Fed mice were used for this experiment. Mice were handled gently and decapitated quickly. Immediately, 0.1 g of liver (approximately) was taken from a fixed lobe of the liver, weighed accurately and transferred to a centrifuge tube containing 3 ml of 30% KOH solution. The tube was placed upright in a boiling water bath for 15 to 20 minutes. When thorough disintegration was ensured, the tube was allowed to cool. Seven ml of 95% ethanol were added to the tube, mixed by tapping and immersed in the water bath until boiling. The tube was allowed to cool at room temperature for about 2 hours. Then the tube was centrifuged and the supernatant fluid was decanted. The precipitate was washed twice with 5 ml portions of 60% alcohol. Last traces of alcohol were expelled by immersing the tube in boiling water bath. The sediment was hydrolysed for 30 minutes in 2 ml of 5 M HCl in a boiling water bath. The hydrolysate was cooled and neutralized with 5 M NaOH using phenolphthalein as indicator and made up to a volume of 10 ml. A 0.5 ml of the solution was taken into a 10 ml graduated test tube. Glucose was estimated by the method of Nelson and Somogyi [229, 230]. One ml of alkaline copper reagent (Solution A. 50 g of anhydrous sodium carbonate, 50 g of Rochelle salt, 40 g of sodium bicarbonate and 400 g of anhydrous sodium sulphate were
dissolved in about 1600 ml of distilled water, and diluted to two litres. And the solution was filtered \textbf{Solution B}. 150 g of copper sulphate was dissolved in distilled water and diluted to one litre. A 0.5 ml of concentrated sulphuric acid was added and mixed. Working solution of alkaline copper reagent was prepared on the very day of its use. Four ml of solution B were placed in a 100 ml graduated cylinder and diluted to 100 ml with solution A and mixed thoroughly) was added and mixed by tapping. The top of the tube was covered with a glass ball and the tube was placed upright in a boiling water bath for 20 minutes. The tube was taken into room temperature and 1 ml of arsenomolybdate colour reagent (100 g of ammonium molybdate was dissolved in 1800 ml distilled water. Concentrated sulphuric acid (84 ml) was added to it with stirring. Twelve g of disodium ortho-arsenate was dissolved in 100 ml distilled water and was added with stirring to the acidified molybdate solution. The mixture was placed in an incubator at 37°C for 12 days. The reagent was stored in a glass-stoppered brown glass bottle) was added in the test tube and then mixed by tapping and then diluted to 10 ml with distilled water. The content in the tube was mixed by inversion. A blank was prepared by carrying out the procedure as described with 0.5 ml of water. A standard was also prepared by carrying out the same procedure with 0.5 ml standard solution (stock solution was prepared by dissolving 1 g anhydrous glucose in 10 ml of 0.2 percent benzoic acid and then the volume was made up to 100 ml with water. Working standard was prepared by dilution 0.5 ml of stock standard to 200 ml with 0.2 percent benzoic acid solution). Optical densities of standard and of unknown were measured at 540 nm and then from the readings the amount of glucose present per sample was calculated.

**ii) Estimation of blood glucose [229, 230]**

A 0.1 ml of heparinized blood was taken in a centrifuge tube. The blood was deproteinized by adding 0.95 ml of barium hydroxide solution (90 g of Ba (OH)$_2$, 8H$_2$O was dissolved in distilled water and diluted to 2000 ml in a graduated cylinder) to it and was mixed by rotation. Then 0.95 ml of zinc sulphate solution
(100 g of ZnSO₄·7H₂O was dissolved in distilled water, diluted to 2000 ml in a graduated cylinder and mixed well) was added to the centrifuge tube and mixed thoroughly. After centrifugation, the supernatant was collected. A 0.5 ml of the supernatant was used for determination of glucose by the Nelson and Somogyi method [229, 230].

iii) Determination of serum aspartate aminotransferase (SGOT) activity [231]

Fresh cold blood was allowed to clot. Serum (free from haemolysis) was collected. Then enzymatic reactions were determined following the procedure given below.

Two tubes were taken, one of experimental and the other for blank. One ml substrate-buffer solution (1.5 g, K₂HPO₄, 0.30 g, KH₂PO₄, 0.030 g, α-oxoglutaric acid and 1.32 g L-aspartic acid were dissolved in less than 100 ml double distilled water. pH was adjusted to 7.4 with 0.4 N NaOH and the solution was diluted to 100 ml with double distilled water) was taken in each of the tubes. A 0.2 ml serum (1:10 dilution with physiological saline) was added to the substrate buffer solution, mixed by inversion and incubated at 37°C for exactly 60 minutes. No serum was added to the blank tube and no incubation was done. After incubation, 1 ml of ketone reagent (20 mg, 2,4-dinitrophenylhydrazine was dissolved in 1 N HCl and the volume was made up to 100 ml) was added to each tube. A 0.2 ml serum was then added to the blank one. The tubes were allowed to stand for 20 minutes at room temperature. Ten ml of 0.4 N NaOH was mixed into both the tubes. After 5 minutes, the optical density of the extent of experimental tube was read against the blank at the wave length, 530 nm in the spectrophotometer. From the standard curve, the unit present in the sample was calculated.

iv) Determination of serum alanine aminotransferase (SGPT) activity [231]

In each of two tubes, one for experimental and the other for blank, 1 ml substrate-buffer solution (1.5 g, K₂HPO₄, 0.20 g KH₂PO₄, 0.030 g α-oxoglutaric acid
and 1.78 g, DL-analine were dissolved in double distilled water and the volume was made up to 100 ml. pH of the solution was checked) was taken. A 0.2 ml serum (1:10 dilution with physiological saline) was added to the substrate-buffer solution, mixed by inversion and incubated at 37°C for exactly 30 minutes. No serum was added to the blank tube and no incubation was done. After incubation, 1 ml of ketone reagent (described before) was added to each tube. 0.2 ml serum was then added to the blank one.

The tubes were allowed to stand for 20 minutes at room temperature. Ten ml of 0.4 N NaOH was mixed into each tube. After 5 minutes, optical density of the content of the experimental tube was read against the blank at 530 nm. From the standard curve the unit present in the sample was calculated.

v) Estimation of total bilirubin and conjugated bilirubin from serum [232]

**Total bilirabin : Test** : Into a test tube, 1.8 ml of water and 0.2 ml of serum was taken. Then 0.5 ml of freshly prepared diazo reagent (10 ml of solution A and 0.3 ml of solution B) was added and mixed.

(Solution A : One gram of sulphanilic acid and 15 ml of concentrated hydrochloric acid/litre of water. This solution keeps indefinitely at room temperature.

Solution B : A 0.5 g of sodium nitrite per 100 ml in water. This solution was kept in the refrigerator and renewed monthly. The characteristic smell of diazo reagent when first-mixed indicate that nitrite has not decomposed).

**Control** : In a test tube, 1.8 ml of water, 0.2 ml of serum and 0.5 ml of diazo blank solution (15 ml of concentrated hydrochloric acid per litre in water) was added.

**Standard** : A 1.8 ml of water, 0.2 ml of bilirubin standard (10 mg per 100 ml water) and 0.5 ml of diazo reagent was taken in another test tube.

**Standard control** : In the last tube, 1.8 ml of water, 0.2 ml of standard and 0.5 ml of diazo blank solution was taken.
To each tube, 2.5 ml of methanol was added and mixed. It was allowed to stand in dark for 30 minutes and color was compared within next 10 minutes at 540 nm.

Conjugated bilirubin: This method is identical except that the 2.5 ml of methanol in the Test and Control were replaced by 2.5 ml of water. It was noted that this only applied to the tubes containing the serum specimens and not the standard and control.

vi) Determination of serum alkaline phosphatase activity [233, 234]

p-Nitrophenyl phosphate was used as the substrate for the determination of the activity of the alkaline phosphatase. p-Nitrophenol liberated by the phosphate forms a yellow anion.

The assay mixture contained one micromole of p-nitrophenyl phosphate in 1 M Tris-buffer pH 8. (4.175 mg of p-nitrophenyl phosphate dissolved in 10 ml of Tris-buffer, 0.1 ml of this was added to the assay mixture. Tris-buffer was prepared by dissolving 6.05 g of Tris hydroxy methyl amino methane in 50 ml of glass distilled water and pH was adjusted to 8 with 1 M HCl). Two tubes were taken for each set of experiment. One tube was marked as ‘sample’ other was marked as control. A 0.1 ml of p-nitrophenyl phosphate and 0.8 ml of Tris-buffer were taken into both the tubes. Test tubes were incubated for 10 minutes at 37°C. A 0.1 ml of diluted serum (serum was diluted 5 times with physiological saline) was added to the sample tube. The solution was mixed properly by inverting the tubes and incubated for 30 minutes at 37°C. A 0.05 ml of 3 N NaOH solution was added to both the tubes and shaken properly to stop the reaction. After this step 0.1 ml of diluted serum was added to the test tube marked ‘Test’. The content of each tube was mixed by inverting several times and the optical densities were taken at 405 nm against blank using buffer and serum.
vii) Determination of free fatty acid of whole blood [235]

Blood was collected from each group of mice separately by cardiac puncture. To each fraction 20 ml of alcohol : ether (1 : 1) mixture was added and kept for overnight. It was then stirred for 2 hours by magnetic stirrer. The content was filtered and the filtrate was kept in cold. The residue was taken into 20 ml of chloroform : methanol (2 : 1) mixture and stirred for 2 hours. Both the filtrates were collected together and evaporated to dryness. Ten ml of alcohol : ether (1 : 1) was added to the dry substance (alcohol-ether mixture was neutralized with 0.1 N KOH using 1 ml of phenolphthelain as an indicator). It was gently heated on water bath until the substance was dissolved. The solution was titrated with 0.1 KOH (5.61 g of KOH dissolved in 1000 ml of distilled water and standardized against 0.1 N oxalic acid using phenolphthelain as an indicator) shaking continuously until a pink colour persisted for 15 seconds. Number of ml of 0.1 N KOH was recorded. A blank of alcohol : ether mixture was titrated with 0.1 N KOH and titre volume was noted. Free fatty acids value per 100 ml of 0.1 KOH calculated from the number of ml of 0.1 N KOH consumed. The acid value is the number of mg of KOH required to neutralize the free fatty acid in 1 g of substance.

viii) Determination of serum cholesterol (estimated by Libermann – Burchard reaction) [236]

A 0.2 ml of serum was taken into the centrifuge tube. Twelve ml of alcohol-ether mixture (9 ml of absolute alcohol and 3 ml of ether) was added. The tube was tightly stoppered and shaken vigorously for about 2 minutes. The tube was placed horizontally to evenly distribute the precipitate along the tube. The tube was kept for 30 minutes with occasional shaking. It was then centrifuged for five minutes, clean supernatant fluid was completely decanted into another tube. This clean fluid was evaporated to dryness in a water bath. The residue after evaporation was dissolved in 5 ml of chloroform. Standard cholesterol was prepared (0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml) with chloroform in five different test tubes for standard curve. Volume was adjusted to 5 ml with chloroform. In another test tube only 5 ml of chloroform was
taken which served as a blank. Two ml of the freshly prepared mixture of acetic anhydride and sulphuric acid (20 ml of anhydride acetic acid and 1 ml of concentrated sulphuric acid) were added to each tube. The solution was mixed and kept in the dark at 25°C for 15 minutes. The resulting green colour was read immediately in the Spectrocolorimeter (systronic M. No. 103) at 680 nm against blank. Quantity of cholesterol (mg/100 ml of serum) was calculated from the standard curve.

ix) Histological studies of liver [237]

Pieces of liver tissues (from left lobes) of sacrificed animals were fixed in 4% Formal (10 ml of 40% formaldehyde made upto 100 ml with normal saline) for overnight. Then the tissues were dehydrated with graded concentration of alcohol, cleared with benzene and embedded with paraffin bath. The paraffin blocks were cut at 5 μm in a microtome and the sections were stained with eosin and hematoxylin and mounted in Canada balsam on slides. Thus the slides were prepared for microscopical observation to study the architecture of liver.

Results and Discussions

δ-Endotoxin caused a depletion in liver glycogen and SGOT and an increase in blood glucose and SGPT level (Table 4.1 and Figure 4.1). The conjugated and total bilirubin content of blood serum also increased significantly (Table 4.1 and Figure 4.2). It was found that serum alkaline phosphatase, cholesterol and free fatty acids of whole blood significantly increased. Liver has a pivotal role in regulation of physiological processes. Liver damage is generally assessed by measuring serum marker enzymes, bilirubin, histopathological changes in the liver, biochemical changes in liver [238]. When liver is damaged, liver enzymes such as glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and
alkaline phosphatase enter into the circulation to large extent. An alteration in the levels of these marker enzymes in the serum is an indicator of liver damage [239]. In hepatocellular disorder increase in SGPT level is found. Liver necrosis also causes increased SGPT level [240]. δ-endotoxin caused hepatocellular disorder or necrosis of liver and thereby increased SGPT level. The contrasting feature of this study is that an inverse relationship between SGPT and SGOT level has been observed after δ-endotoxin administration. Normally during liver necrosis/damage concentration of SGPT and SGOT increase [238]. Decrease of SGOT level has however been reported in untrained human subjects due to stress condition [241].

Table 4.1. Effect of δ-endotoxin on liver functions and metabolism in mice (Means ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Saline Control (10a)</th>
<th>δ-Endotoxin (kitu(^b)/Kg)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>70 (15(^b))</td>
</tr>
<tr>
<td>Glucose (mg%)</td>
<td>131.5 ± 4.2</td>
<td>152.4 ± 4.3**</td>
</tr>
<tr>
<td>Glycogen (mg/g liver)</td>
<td>25.0 ± 1.2</td>
<td>20.4 ± 1.2*</td>
</tr>
<tr>
<td>SGPT (IU/L)</td>
<td>44.6 ± 2.0</td>
<td>52.8 ± 2.3*</td>
</tr>
<tr>
<td>SGOT (IU/L)</td>
<td>84.4 ± 2.5</td>
<td>73.7 ± 2.2**</td>
</tr>
<tr>
<td>SAP (IU/L)</td>
<td>46.1 ± 2.1</td>
<td>53.9 ± 1.9*</td>
</tr>
<tr>
<td>Conjugated Bilirubin (mg%)</td>
<td>3.1 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>Total Bilirubin (mg%)</td>
<td>7.8 ± 0.3</td>
<td>8.6 ± 0.2</td>
</tr>
<tr>
<td>Cholesterol (mg%)</td>
<td>134.6 ± 5.6</td>
<td>148.7 ± 5.0</td>
</tr>
<tr>
<td>Free Fatty acids (mEq/L)</td>
<td>351.8 ± 7.0</td>
<td>369.9 ± 5.2</td>
</tr>
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a = No. of animals  
\(b = \) kilo international toxic unit

\* p < 0.05,  \** p < 0.02,  \*** p < 0.001
Fig. 4.1. Effect of δ-Endotoxin of Liver Glycogen, Blood Glucose level, SGPT, SGOT and SAP level in mice
Fig. 4.2. Effect of 8-Edotoxin on Bilirubin content of serum Cholesterol and Fatty acid level of the whole blood in mice.
Fig. 4.3. Showing normal histological feature of liver x 600

Fig. 4.4. Showing congested central vein with a partial degeneration of hepatic cells x 600
Fig. 4.5. Showing hepatic cell degeneration along with congestion of central vein is apparent in this group x 600
Depletion in liver glycogen content and an increase of blood glucose may be due to increased glycogenolysis or adrenal hyperactivity. It may also be due to an increased activity of phosphorylase or low peripheral utilization of glucose. Hyperbilirubinemia caused by over production of bilirubin due to excessive haemolysis and inability of liver to adequately sequester this pigment from blood. Cirrhosis of liver causes an increase of bilirubin content in blood. Elevation of total and conjugated bilirubin indicates a post hepatic obstruction generally a partial or total blockage of larger bile passages particularly the common bileduct resulting a reflux of bilirubin [242, 243]. Sometimes pulmonary infarction may cause elevation of bilirubin [244].

Alkaline phosphatase is synthesized at various sites of body, principally by the osteoblast and also by liver and is excreted by into bile. If there is obstruction to the biliary outflow the enzyme is regurgitated into plasma. Damage liver cells may also liberate more enzyme [245]. In damaged liver, fatty acids can not be oxidized to acetoacetates and ultimately elevated in plasma [245]. Liver insufficiency and anemia may produce lipoaemia [245] and plasma cholesterol level increases with lipoaemia. The combination of lipoaemia and hypercholesteremia is commonly seen in diabetes, nephrosis and bilary cirrhosis. In case of δ-endotoxin treatment, alteration of the liver cells may responsible for the enhancement of alkaline phosphatase, free fatty acids and cholesterol in blood.

Histologically it has been found that the normal architecture of the hepatic cells were degenerated by δ-endotoxin. The Figure 4.4 and 4.5, in comparison to control group figure 3.3, reveal the histology of liver with dilated and congested central vein and normal portal triads but with sinusoidal dilation and congestion. The histological features which indicate the degeneration of hepatic cells, strongly support the biochemical alterations of liver function tests.