BIOCHEMICAL MARKER STUDIES OF ACUTE DRUG INDUCED LIVER INJURY IN RAT

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ABSTRACT: Liver is an organ of paramount importance which plays an essential role in the metabolism of foreign compounds entering into the body. AST, ALT, ALP, GGT, Bilirubin, proteins, lipids etc. are most sensitive parameters which are considered as indices for the diagnosis of liver diseases. Acid phosphatase plays a vital role in the autolytic degradation of dead cells and Alanine Aminotransferase is the liver specific enzyme in rat. The detection of liver injury in the clinical setting is often accomplished by the use of a battery of tests for liver functions. These tests include Serum Aspartate Transferase and ALT, which measure the integrity of the hepatocyte and the sinusoidal plasma membrane; serum albumin and hepatic clotting factors measure the biosynthetic capacity and Serum Bilirubin, ALP and γ-glutamyl transferase as a measure of biliary function.

KEYWORDS: Drug-Induced Liver Injury (DILI), Antibiotics, Liver, Xenobiotics, Hepatotoxicity, Biomarkers.


INTRODUCTION: The liver carries out four main functions in protein metabolism: formation of plasma proteins, amino acid interconversion, de-amination of amino acids and urea synthesis. The liver manufactures a broad spectrum of proteins including many clotting factors, albumin, thyroid binding globulin, VLDL. Since Glutathione is essential for cellular detoxification of many toxic Xenobiotic substances it is an essential enzyme. The liver undergoes dramatic changes in structure and function during development. The developmental changes that occur in the liver determine the rate and metabolic pathways used in the disposition of drugs and other Xenobiotic. Biochemical markers are increasingly used in ecological risk assessment to identify the incidence of exposure and effects caused by Xenobiotic (Otitoju and Onwurah, 2007). Some toxicants cause direct injury to liver and others convert the chemicals into toxic substance through metabolic conversion. The classification may focus on the source and the chemical class of the toxicant, on the circumstances of exposure on the type of hepatic lesion produced, on the cell structure damaged or on the molecular or cellular mechanisms involved. Severe case may result in fulminant liver failure. Due to the presence of different types of cells blood has varied functions and analysis of its components helps in evaluating the abnormal conditions which create pathological conditions. Hepatic toxicity as a result of drugs and environmental toxins presents a wide spectrum of clinical diseases (Bhaskar, 2012).

Chemical Structure of Tetracycline

PATHOLOGY OF TETRACYCLINS: Microvesicular steatosis was the characteristic feature of treatment with intravenous or large oral doses of tetracycline, whereas cholestasis was the predominant clinico-pathological pattern with oxytetracycline and minocycline. Long-term minocycline used as a treatment for acne has also been associated with autoimmune hepatitis, characterized by antinuclear autoantibodies, a lupus-like syndrome, fatigue, rash and arthralgia and hypersensitivity syndrome. Zimmeron (1978) revealed that the therapeutic agents like Acetaminophen, Papaverine, Tetracyclins and Nitrofurantoin are Hepatotoxic which taken in over dose or in case of poisoning.

The hepatotoxicity of therapeutic agents and pharmaceutical chemicals has become an area of intense research interest (Bhaskar, 2015). Number of areas considered for the study were interference of antibiotic with experiments, antibiotic toxicity, route of administration, effects of formulation on bioavailability, antibiotic prophylaxis, use of combinations of antibiotics, misuse of antibiotics, regulatory approval of antibiotic use in animals, Sources of information on antibiotic reactions and dose, extrapolation of dose information from other sources.

The parameters of the study life span, adult body weight, metabolic rate, water intake and blood parameters like glucose, protein, and enzymes.

MATERIALS AND METHODS: In accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.
EXPERIMENTAL PROCEDURE:

Sample Collection: The rats were killed and the liver quickly removed and examined for the liver injury (Oberdorster, 2000). After 60 days of treatment, the animals were fasted for 12 hours, anaesthetized with pentobarbital sodium (35mg/kg) and sacrificed by cervical decapitation. Blood samples were collected through cardiac puncture into lithium heparin specimen bottles for biochemical analysis. The blood was collected in tubes containing mixture of potassium oxalate and sodium fluoride as anticoagulant for the biochemical analysis. Liver dissected out, washed in ice-cold saline, patted dry and weighed. 10% tissue homogenate prepared from liver was used for various biochemical estimations.

Preparation of Tissue Extract: After the end of the experimental period, the rats were starved (Deprived of food) for 18 hours, overnight and sacrificed by cervical dislocation. Liver were dissected out, blotted off blood, rinsed in phosphate buffered saline (pH 7.4) and 10% tissue homogenate were prepared.

EXPERIMENTAL GROUPS:

1. Group-I - Control Rats treated with normal feed.
2. Group-II - Test-1 Rats fed with 0.200 mg per kg Tetracyclin
3. Group-III - Test-2 Rats fed with 0.400 mg per kg Tetracyclin
4. Group-IV - Test-3 Rats fed with 0.600 mg per kg Tetracyclin
5. Group-V - Test-4 Rats fed with 0.800 mg per kg Tetracyclin
6. Group-VI - Test-5 Rats fed with 1.000 mg per kg Tetracyclin

Detection of Chronic Toxicity: Two classes of methods are available to detect chronic toxicity from these substances in the liver: structural and functional. The structural methods involve histological examination of tissues by microscopic techniques specifically developed to measure the structural or compositional changes associated with exposure to these substances. The sensitivity of these procedures is usually limited by the availability of microscopy equipment and trained histopathologists. Hepatotoxicity induced in rats by administering various agents. In addition to biochemical parameters, the change in the histopathology of the liver treated rats were examined.

Preparation of Haemolysate: The blood for haemolysate preparation was collected in bottles containing the anticoagulants whereas the blood from the serum was collected separately. After 30 minutes, blood was centrifuged at 2000 x g for 15 minutes in cold centrifuge. After centrifuge the blood plasma and buffy coat were removed with Pasteur pipette. The remaining plasma and buffy coat were removed by resuspending the erythrocytes in the volume of ice-cold 0.9% NaCl solution and centrifuged at 2000 x g for 10 minutes. The erythrocytes were then washed thrice with the same volume.

After washing the erythrocytes were resuspended in equal volume of cold 0.9% NaCl

About 0.2ml of this suspension was added to 1.8ml of β-mercaptoethanol EDTA (0.27M) made upto a volume of 1 litre in a glass tube. Capped the tube and immersed in freezing mixture until it was completely frozen and was then thawed by placing the tube into the beaker containing water at room temperature. This process was repeated thrice to achieve maximum lysis of erythrocytes. The lysis was then stored at -4°C till further analysis. The haemolysate thus obtained was 1:20 dilution.

Preparation of Liver Homogenate: The liver tissue was ground in cold mortar and pestle or homogenizer. Homogenate was kept for 15 minutes under cold conditions and collected the clear homogenate from the top for studies.

Estimation of Haemoglobin:
(Varley, 1975).
Haemoglobin was treated with Drabkin’s solution.

REAGENTS: Drabkin's solution (Ferricyanide-cyanide reagent)
Potassium Ferricyanide - 200 mg
Potassium cyanide - 50 mg
Potassium dihydrogen phosphate - 140 mg

Dissolved in 800ml water and added, 1 ml Noridet P40 or 0.5 ml streox S.E. Made upto 1 litre with water and checked pH between 7.0 and 7.4(7.2). The reagents can be kept for several months in a dark polyethylene bottle between 4 and 20°C.

PROCEDURE: Added 0.02ml blood into 4.0 or 5.0ml reagent and stood atleast 4minutes at RT and read against water blank at 540nm. Read absorbance of standard solution against Drabkin solution.

Grams Hb/100 ml = Reading of unknown×Dilution factors×conc. of std.

Reading of Standard 1000
Determination of Proteins (Lowry, et al, 1951). Pippeted out about 0.2ml of distilled water (For blank), working standard and test solutions in different test tubes, made up all the test tubes upto 2ml using 0.1 N NaOH. To all tubes added 5ml of alkaline copper reagent and mixed well and kept for 15min. Then 0.5 ml of Fohlin’s phenol reagent was added to all tubes and kept for 30min. The optical density was read at 675nm.

LIVER MARKER ENZYMES:
1ml of buffered substrate is added to two test tubes marked test and control. 0.2ml of serum is added to test only. In blank 1.2ml of distilled water is added and to standard; 1 ml of working standard taken along with 0.2ml of distilled water. All the tubes were then incubated for 60minutes. After the incubation 0.2ml of the serum is added to control and 1 ml DPNHi is added to all the test tubes and kept for 20minutes at room temperature. 10ml NaOH is also added and mixed well. The absorbance is read after 5 minutes at 500-550.
b. ALT (Alanine transaminase) (Mohun and Cook, 1957). 1 ml of buffered substrate is added to two test tubes marked test and control. 0.2 ml of serum is added to test and to blank. 1.2 ml of distilled water is added and to standard; 1 ml of working standard taken along with 0.2 ml of distilled water. All the tubes were then incubated for 30 minutes. After the incubation 0.2 ml of the serum is added to control. 1 ml DPNH is added to all the test tubes and kept for 20 minutes at room temperature. 10 ml NaOH is also added and mixed well. The absorbance is read after 5 minutes at 500-550.

c. ALP (Alkaline phosphatase) (Varley, 1988). 2 ml of the buffered substrate was added to 2 ml test tubes labelled as test and control. Incubate for 5 minutes at 37°C in water bath. Add 0.1 ml serum to test only and then incubate the two test tubes at the waterbath for 15 minutes. Add 0.8 ml NaOH and 1.2 ml of NaHCO₃ to test tubes. Add 0.1 serum to the control. Add 1 ml of 4-aminophytotic and 1 ml of KFeCn to both the tubes and read at 520 nm. For standard pipette 1 ml working standard and 1.1 ml buffer and after 15 minutes incubate at 37°C add 0.8 ml NaOH, 1.2 ml of NaHCO₃ followed by 4-aminophytotic and KFeCn as in step-1. For blank pipette 1 ml distilled water and add 102 ml buffer.

d. GGT (γ-Glutamyl Transferase) (Grover lock, 1980). 100 μl serum & 1 ml buffer was warmed to 37°C. The reaction was started by adding 0.1 ml substrate, mixed and monitored the reaction continuously at 405nm in 1 cm cuvette so as to obtain the change in absorbance/ minutes.

**ANTIOXIDANTS:**

a. Reduced Glutathione (Patterson & Lazarrow, 1955). Tissues were homogenized in phosphate buffer. The reaction mixture containing 50 μl tissue extract, 50 μl alloxan, 50 μl phosphate buffer and 50 μl NaOH (0.5N) was incubated at 25°C for 6 minutes. The reaction was stopped by the addition of 50 μl 1N NaOH. Absorbance was noted at 305 nm in a quartz cuvette of 1 cm length in a spectrophotometer. A control tube also maintained using phosphate buffer instead of extract. The reduced glutathione level was expressed as GSH/g tissue weight.

**ANTIOXIDANT ENZYMES:**

a. Glutathione-s-Transferase (Beutler, 1986). Reagent ‘a’ (200 μl) and reagent ‘b’ (20 μl) were added to two tubes named blank and system. 730 μl of distilled water was added to blank and add 680 μl of distilled water was added to system and both the tubes were incubated at 37°C for 10 minutes. Afterwards added 50 μl of reagent ‘c’ to both the tubes and mixed well. 50 μl of hemolysate was added to the system and the change in absorbance was recorded at 340 nm.

b. Glutathione reductase (Beutler, 1986). The reaction mixture contained 50 μl Tris-HCl EDTA buffer and 10 μl 1:20 hemolysate. Also added 890 and 790 μl of distilled water to blank and system respectively. Both the tubes were incubated at 37°C for 10 minutes. After incubation added 100 μl oxidized glutathione to the system and both the tubes were reincubated at 37°C for 10 minutes. 50 μl NADPH was added to both tubes and the change OD was recorded at 340 nm.

c. Glutathione peroxidase (Rotruck, 1984). To 0.2 ml of Tris buffer, 0.2 ml EDTA, 0.1 ml Sodium azide and 0.5 ml sample homogenate, were added and mixed well. To this mixture 0.2 ml of GSH followed by 0.1 ml of H₂O₂ solution was added. The contents were mixed well and incubated at 37°C for 10 minutes along with a control containing all reagents except tissue homogenate. After the reaction was arrested by the addition of 0.5 ml of 10% TCA. Tubes were centrifuged and supernatant was assayed for GSH. 1 ml of the supernatant from above test tubes was taken. 0.5 ml of Ellmann’s reagent 19.8 mg DTNB/100 ml of 0.1% Sodium citrate and 3 ml of phosphate buffer were added. The yellow color developed was read at 412 nm.

**Estimation of lipid peroxide (Beutler, 1986)**: Liver homogenized with 40M Tri-HCl buffer (pH 7.0) and centrifuged at 3000 rpm for 10 minutes to get a clear supernatant which was transferred to ice-cold container for further analysis.

<table>
<thead>
<tr>
<th>Table 1: Estimation of Hemoglobin after 60 days of Tetracycline treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group - 1 Normal-C</td>
</tr>
<tr>
<td>Group – 3 T2</td>
</tr>
</tbody>
</table>

a. Statistical difference with control group at P < 0.05.

b. Statistical difference with test group at P < 0.05.

<table>
<thead>
<tr>
<th>PERIOD</th>
<th>Group-1 Control</th>
<th>Group-2 T1</th>
<th>Group-3 T2</th>
<th>Group-4 T3</th>
<th>Group-4 T4</th>
<th>Group-5 T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>INITIAL</td>
<td>11.3±3.1 b</td>
<td>10.1±3.4 a</td>
<td>10.2±3.4 a</td>
<td>10.2±3.2 b</td>
<td>10.3±3.4 a</td>
<td>10.1±3.3 a</td>
</tr>
<tr>
<td>15 DAYS</td>
<td>10.3±3.4 b</td>
<td>10.2±3.5 b</td>
<td>10.2±3.4 b</td>
<td>10.3±3.2 b</td>
<td>10.1±3.5 b</td>
<td>11.3±3.8 b</td>
</tr>
<tr>
<td>30 DAYS</td>
<td>10.2±3.5 b</td>
<td>10.4±3.5 b</td>
<td>10.5±3.2 b</td>
<td>11.3±3.5 b</td>
<td>10.3±3.3 b</td>
<td>11.3±3.5 b</td>
</tr>
<tr>
<td>45 DAYS</td>
<td>10.1±3.1 b</td>
<td>10.5±3.8 b</td>
<td>10.6±3.7 b</td>
<td>11.5±3.7 b</td>
<td>10.5±3.5 b</td>
<td>10.3±3.4 b</td>
</tr>
<tr>
<td>60 DAYS</td>
<td>10.3±3.1 b</td>
<td>11.0±3.2 b</td>
<td>11.3±3.4 b</td>
<td>11.8±3.6 b</td>
<td>11.3±3.2 b</td>
<td>11.4±3.2 b</td>
</tr>
<tr>
<td>90 DAYS</td>
<td>10.0±3.5 b</td>
<td>10.4±3.5 b</td>
<td>11.0±3.4 b</td>
<td>12.0±3.5 b</td>
<td>10.5±3.5 b</td>
<td>11.3±3.5 b</td>
</tr>
</tbody>
</table>

**Table 2: Weight of liver in experimental rats after 60th day of Tetracycline treatment**

a. Statistical difference with control group at P < 0.05.

b. Statistical difference with test group at P < 0.05.
The Values are average for six rats in each group and are expressed in grams± SEM.

Group-1 Control, Group-2 to 6 are tests.

a. Statistical difference with control group at P <0.05.
b. Statistical difference with test group at P <0.05

**Graph 1:** Weight of liver in experimental rats after 60th day

**Table 3:** Biochemical Tests – Protein, Bilirubin, SGOT, SGPT, GGT and ALP after 60 days of treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>T</th>
<th>Total Protein g/dl</th>
<th>Bilirubin g/dl</th>
<th>SGOT (AST) IU/L</th>
<th>SGPT (ALT) IU/L</th>
<th>GGT IU/L</th>
<th>ALP IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td>6.40 ±0.24</td>
<td>1.26 ±0.04</td>
<td>37.09 ±0.96</td>
<td>27.76 ±0.95</td>
<td>3.62 ±0.09</td>
<td>84.31 ±2.12</td>
</tr>
<tr>
<td>Group 2</td>
<td>T1</td>
<td>6.83 ±1.84</td>
<td>1.092 ±0.84</td>
<td>32.06 ±1.84</td>
<td>26.88 ±1.84</td>
<td>3.65 ±0.09</td>
<td>86.67 ±1.09</td>
</tr>
<tr>
<td>Group 3</td>
<td>T2</td>
<td>6.80 ±3.46</td>
<td>1.090 ±0.74</td>
<td>32.63 ±1.84</td>
<td>27.08 ±2.74</td>
<td>3.11 ±0.15</td>
<td>89.11 ±1.21</td>
</tr>
<tr>
<td>Group 4</td>
<td>T3</td>
<td>6.78 ±3.35</td>
<td>1.008 ±0.74</td>
<td>30.58 ±1.84</td>
<td>27.38 ±1.85</td>
<td>3.01 ±0.08</td>
<td>85.33 ±3.21</td>
</tr>
<tr>
<td>Group 5</td>
<td>T4</td>
<td>6.87 ±4.14</td>
<td>1.277 ±0.34</td>
<td>35.23 ±1.84</td>
<td>28.68 ±1.83</td>
<td>3.66 ±0.08</td>
<td>84.12 ±1.50</td>
</tr>
<tr>
<td>Group 6</td>
<td>T5</td>
<td>6.97 ±3.12</td>
<td>1.428 ±0.84</td>
<td>31.02 ±1.84</td>
<td>29.71 ±1.87</td>
<td>3.60 ±0.10</td>
<td>84.30 ±1.80</td>
</tr>
</tbody>
</table>

**Graph 2:** Biochemical Changes in experimental rats after 60th day

**Significance**: There was an increase in protein in all the groups or extend when

<table>
<thead>
<tr>
<th>Group</th>
<th>Glutathione–S-transferase</th>
<th>Reduced Glutathione (µ g /liver)</th>
<th>Lipid Peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>1909.5 ±45.5</td>
<td>9321.3 ±683.3</td>
<td>86.0 ±14.50</td>
</tr>
<tr>
<td>Group 2</td>
<td>T1</td>
<td>1950.3 ±45.4</td>
<td>9346.5 ±962.6</td>
</tr>
<tr>
<td>Group 3</td>
<td>T2</td>
<td>1940.3 ±75.4</td>
<td>9386.5 ±862.6</td>
</tr>
<tr>
<td>Group 4</td>
<td>T3</td>
<td>1710.3 ±47.4</td>
<td>9465.6 ±863.6</td>
</tr>
<tr>
<td>Group 5</td>
<td>T4</td>
<td>1629.3 ±45.4</td>
<td>9826.5 ±826.8</td>
</tr>
<tr>
<td>Group 6</td>
<td>T5</td>
<td>1632.3 ±65.4</td>
<td>9346.8 ±802.8</td>
</tr>
</tbody>
</table>

**Table 4:** Biochemical Tests after 60 days of treatment

The Values are average for six rats in each group and are expressed in grams± SEM.
Group-1 Control, Group-2 to 6 are tests.

a. Statistical difference with control group at P < 0.05.

b. Statistical difference with test group at P < 0.05.

**Graph-3**: Biochemical Changes in experimental rats after 60th day

**Significance**: There was an increase in Glutathione in all the groups or extend when compared to the normal which was found to be significant.

**HISTOPATHOLOGY OF RAT LIVER ON EXPERIMENTS**

Liver tissue of Albino rats measuring 5×5×2cm

**Group-1 Normal**
Liver tissue sections show normal appearing Hepatocytes and sinusoids.

**Group-2 Test-1**
The central veins are dilated and the portal triad shows focal infiltrates of lymphocytes

**Group-3 Test-2**
Liver tissue sections shows multiple areas of focal necrosis with mononuclear cells. Infiltrates marked central veins congestion and periportal fibrosis.

**Group-4 Test-3**
Liver show multiples foci cellular necrosis, lymphocytic infiltrates and periportal fibrosis and periportal fibrosis. The central veins show congestion and mild haemorrhage.

**Group-5 Test-4**
Liver tissue areas shows mild haemorrhage with necrosis and other pathological changes.

**Group-6 Test-5**
Liver tissue sections shows multiple areas of focal necrosis, lymphocytic infiltrates and periportal fibrosis. The central vein shows congestion and mild hemorrhages
DISCUSSION: Liver is a major entry organ of Xenobiotic and potential target both for the chemicals circulating in the blood stream. Many Xenobiotics are capable of causing some degree of liver injury. The rat animals in the experimental groups were treated for drugs, as chemicals (Xenobiotics) may affect liver function which stimulate the activity of microsomal enzymes (eg. cyt. P450), a process known as enzyme induction. This is important in determining the degree of hepatotoxicity in the animal study (Conney, 1967). Liver damage is detected by the measurement of the activities of serum enzymes like AST, ALT, ALP, GGT which has been released into the blood from damaged cells, which are indicators of hepatic cell damage. It is the total concentration of antibiotics in mg/kg which administered to animal upto 60 days of experiments. The toxicity of many chemicals results from their metabolic conversion to derivatives that can alter tissue macromolecules by the process of metabolic activation (Mitchell, 1975). All forms of histological injury ranging from cholestasis of amoxicillin to autoimmune hepatitis to telithromycin (Chang and Sciano, 2001).

CONCLUSION: Drugs and other exogenous compounds may affect the liver in various ways (Kshirsagar, 2009). Drug hepatotoxicity may involve metabolism to toxic, reactive intermediates and covalent interactions with cell constituents, interference, with membrane transport or with cellular biochemistry such as protein synthesis, or immunological mechanisms. The occurrence of hepatic damage may be modified by differences in immune responsiveness and genetic, dietary and other factors. Various clinical therapies should involve drug induced hepatotoxicity as an important parameter.

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REFERENCES: