ASSESSMENT OF BIOCHEMICAL PARAMETERS IN DRUG INDUCED LIVER TOXICITY AGAINST RAT HEPATOCYTES

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ARTICLE INFO

Article history
Received 07/11/2013
Available online 30/11/2013

Keywords
Hepatocytes,
ASAT, LDH,
Toxicity and
Hepatitis

ABSTRACT
Assessment of liver can be made by estimating the activities of ASAT, ALAT, ALP and LDH, which are enzymes originally present in higher concentration in cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage. Many toxins target the liver and cause hepatotoxic effects that can be observed through some biochemical parameters. Impairment of the liver generally occurs from excessive exposure to xenobiotics, alcohol, chemotherapeutic agents, virus and protozoan infections. Depending upon the severity of toxicant insult, hepatic cell injury can lead from acute to chronic hepatitis, which if left untreated can result in cirrhosis or malignant lesions. In this paper we study of the effect of selected drugs against each toxicant by estimating the biochemical parameters using hepatocytes.

Please cite this article in press as Mandeep Kaur et. al. Assessment of biochemical parameters in drug induced liver toxicity against rat hepatocytes. Indo American Journal of Pharm Research. 2013:3(12).

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INTRODUCTION
The liver is of vital importance in intermediary metabolism and in detoxification and elimination of toxic substances. The liver is often affected by a multitude of environmental pollutants and drugs, all of which place a burden on this vital organ and can damage and weaken it, eventually leading to diseases like hepatitis or cirrhosis. Paracetamol's hepatotoxicity is caused by its reactive metabolite N-acetyl-p-benzoquinone imines (NAPQI), which causes oxidative stress and glutathione (GSH) depletion. Paracetamol toxicity is due to the formation of toxic metabolites when a part of it is metabolized by cytochrome P450. Introduction of cytochrome or depletion of hepatic glutathione is a prerequisite for paracetamol-induced hepatotoxicity. In spite of tremendous strides in modern medicine, the treatment of liver disorders is inadequate and many formulations containing herbal extracts are used for regeneration of hepatic cells and for protection of the liver against damage.

Hepatic damage is associated with distortion of its metabolic functions and it is still a major health problem. Unfortunately many synthetic drugs used in the treatment of liver diseases are inadequate and also cause serious side effects. In view of severe undesirable side effects of synthetic agents, there is growing interest in evaluating traditional herbal medicines that are claimed to possess hepatoprotective activity. A single drug cannot be effective for all types of severe liver diseases. Therefore, an effective formulation using indigenous medicinal plants has to be developed with proper pharmacological experiments and clinical trials.

Assessment of biochemical parameters
The biochemical parameters, such as total protein was estimated by the method of Gornall. The total cholesterol was estimated by the method of Wybenga. The total bilirubin was estimated by Method of Malloy and Evelyn. Triglycerides were estimated by the method of Fossati and Lorenz. Urea concentration was determined by the method of Bousquet. Immediately after sacrificing the animal, the liver was excised from the animals, washed in ice-cold saline, and the weight of the liver was recorded.

Silymarin is a polyphenolic flavonoid isolated from the fruit and seeds of the milk thistle (Silybum marianum). Various studies indicate that silymarin exhibits strong antioxidant activity and shows protective effects against hepatic toxicity induced by a wide variety of agents by inhibiting lipid peroxidation. In the present study it was noted that the administration of paracetamol decreased the levels of total protein, total cholesterol, and triglycerides. These parameters were maintained at normal levels in the BHEE-treated animals. BHEE treatment showed a protection against the injurious effects of paracetamol that may result from the interference with cytochrome P450, resulting in the hindrance of the formation of hepatoxic free radicals. The site-specific oxidative damage in some susceptible amino acids of proteins is now regarded as the major cause of metabolic dysfunction during pathogenesis. Attainment of near normal level of protein, cholesterol, and triglyceride levels in paracetamol-intoxicated and BHEE-treated rats confirms the hepatoprotective effect of the plant extract.

The marked elevations in bilirubin and urea levels in the serum of Group II paracetamol-intoxicated rats were significantly decreased in the BHEE-treated animals. Bilirubin is the conventional indicator of liver diseases. These biochemical restorations may be due to the inhibitory effects on cytochrome P450 and promotion of its glucuronidation.

2. MATERIALS & ANIMALS
Colony bred Wistar strain adult albino rats (150g-200g) of either sex were used. All the animals were maintained under standard husbandry conditions with food and water ad libitum.

Drug samples and toxicants
Catechin, L-ornithin-L-aspartate, L-ornithin and silymarin pure samples were procured from Sigma chemicals Co., St. Louis, MO, USA. Lecithin pure sample was procured from Lipoid, Germany.

D-Galactosamine, paracetamol, isoniazid, rifampicin and pyrazinamide pure samples were procured from Sigma chemicals Co., St. Louis, MO, USA. Alcohol and carbon tetra chloride (CCl4) was procured from Merck Ltd., Mumbai and Qualigens Fine chemicals, Mumbai.

METHOD
Preparation of Freshly isolated rat hepatocytes: The availability of methods for isolation of large quantities of intact cells had made isolated hepatocytes culture a favorite experiment system for pharmacological, toxicological and biochemical research. The pioneering studies have established the superiority of collagenase treatment over the older mechanical and chemical methods of liver cell preparation and the introduction of...
enzymatic liver perfusion techniques increased the efficiency of tissue dissociation to such an extent to allow most of the liver tissue to be converted to a suspension of intact cells. In later studies, a quantitative liver dissociation assay to study the methodological parameters of collagenase perfusion established that the most optimal and reproducible results are obtained by a two-step procedure. In the first step the liver is subjected to non-recirculating perfusion with calcium free buffer or with a calcium chelator like EDTA, causing irreversible separation of desmosomal cell contacts. In the second step liver is perfused with collagenase to dissolve the extra cellular matrix, calcium being added back to ensure maximal enzyme activity. This optimal treatment dissociates the liver completely within 10–15 mins, that is, sufficiently rapid to obviate the need for continuous oxygenation during perfusion [23].

3. PROCEDURE
a. The HEPES buffer and collagenase solution were warmed in a water bath
   Usually (38oC-39oC to achieve 37oC in the liver)
b. The pump flow rate was adjusted to 30ml/min.
c. The rat (180-200gms) was anaesthetised by intra peritoneal administration of
   Thiopental sodium 45mg/kg b.w.
d. The abdomen was opened and a loosely tied ligature was placed around the portal vein approximately 5mm from the
   liver, and the cannula was inserted up to the liver and then the ligature was tightened, and heparin (1000 IU) was
   injected into the femoral vein.
e. Sub hepatic vessels were rapidly incised to avoid excess pressure and 600ml of calcium free HEPES buffer was
   perfused at a low rate of 30ml/min for 20 minutes. The liver swells during this time slowly changing color from
   dark red to greyish white.
f. 300ml of collagenase solution were perused at a flow rate of 15ml/min for 20 minutes during which the
   lobes swell.
g. The lobes were removed and washed with HEPES buffer, after disrupting the Glison capsule.
h. The cell suspension was centrifuged at 1000 RPM to remove the collagenase, damaged cells and non-parenchymal
   cells.
   I. The hepatocytes were collected in Ham’s F12 medium enriched with
      0.2%bovine albumin, 10 µg/ml bovine insulin and 0.2% of dexamethasone.

In vitro estimation of biochemical parameters against D-galactosamine intoxicated rat hepatocytes
a. The hepatocytes isolated were incubated for 30 minutes at 37oC for stabilization.
b. The cells were then diluted in F12 coons modified medium to obtain a cell count 5x10^5cells/ml.
c. 100 ml of this cell suspension was seeded in 96 well plates in each well.
d. After 2 hours of pre-incubation, the medium was replaced with fresh medium.
e. Then the hepatocytes were pretreated with extracts for one hour before Galactosamine (30 mM) - induced treatment
   (100µl of different extract concentration and 100µl of D- galactosamine into each well).
g. After incubation, the toxicant and drug treated cell suspensions were pooled into eppendorf tubes and centrifuged at
   4000 rpm for 10 -15 min.
h. Supernatant was collected and the following enzyme levels were determined
   • ASAT (Asparate Aminotransferase)
   • ALAT (Alanine Aminotransferase)
   • ALP (Alkaline Phosphatase)
   • LDH (Lactate dehydrogenase) [24]

In vitro estimation of biochemical parameters against alcohol intoxicated rat hepatocytes [25]
Procedure same as above. Except step e, which is explained below?
   • The hepatocytes were pretreated with drug samples for one hour before alcohol (60 mM)
   - induced treatment (100µl of different drug sample and 100µl of alcohol into each well).
**In vitro** estimation of biochemical parameters against CCl₄ intoxicated rat hepatocytes [26]

Procedure same as above except step e, which is explained below.

- The hepatocytes were pretreated with drug samples for one hour before CCl₄ (15 mM) - induced treatment (100µl of different drug sample and 100µl of CCl₄ into each well).

**In vitro** estimation of biochemical parameters against paracetamol intoxicated rat hepatocytes [27]

Procedure same as above except step e, which is explained below.

- The hepatocytes were pretreated with different drug samples for one hour before paracetamol (50 mM) - induced treatment (100µl of different drug sample and 100µl of paracetamol into each well).

**In vitro** estimation of biochemical parameters against INH: RIF: PYZ intoxicated rat hepatocytes [28]

Procedure same as above except step e, which is explained below.

- The hepatocytes were pretreated with different drug sample for one hour before INH: RIF: PYZ (90 µg/ml) - induced treatment (100µl of different drug sample and 100µl of INH: RIF: PYZ into each well).

RESULTS

Studies using freshly isolated rat hepatocytes

**Graph 1:** Effect of treatment of selected drugs on the biochemical parameters of D-GalN intoxicated freshly isolated rat hepatocytes
A significant increase in the levels of ASAT, ALAT, ALP and LDH (P < 0.001) was observed in hepatocytes exposed to DGaN when compared to normal cells. These cells, when pretreated with the hepatoprotective drugs showed a significant restoration of the altered biochemical parameters towards normal (P < 0.001, when compared to DGaN treated cells).

**Graph 2:** Effect of treatment of selected drugs on the biochemical parameters of alcohol intoxicated freshly isolated rat hepatocytes

*Average of six independent determinations, values are mean ± S.E.M. a = P < 0.001, when compared to normal cells. z = P < 0.001, when compared to alcohol treated cells. A significant increase in the levels of ASAT, ALAT, ALP and LDH (P < 0.001) was observed in hepatocytes exposed to alcohol when compared to normal cells. These cells, when pretreated with the hepatoprotective drugs showed a significant restoration of the altered biochemical parameters towards normal (P < 0.001, when compared to alcohol treated cells)
**Graph 3:** Effect of treatment of selected drug on the biochemical parameter of CCl4 intoxicated freshly isolated rat hepatocytes

A significant increase in the levels of ASAT, ALAT, ALP and LDH (P < 0.001) was observed in hepatocytes exposed to CCl4 when compared to normal cells. These cells, when pretreated with the hepatoprotective drugs showed a significant restoration of the altered biochemical parameters towards normal (P < 0.001, when compared to CCl4 treated cells).
Graph 4: Effect of treatment of selected drugs on the biochemical parameters of Paracetamol intoxicated freshly isolated rat hepatocytes

-Average of six independent determinations, values are mean ± S.E.M. a = P < 0.001, when compared to normal cells. z = P < 0.001, when compared to paracetamol treated cells. A significant increase in the levels of ASAT, ALAT, ALP and LDH (P < 0.001) was observed in hepatocytes exposed to paracetamol when compared to normal cells. These cells, when pretreated with the hepatoprotective drugs showed a significant restoration of the altered biochemical parameters towards normal (P < 0.001, when compared to paracetamol treated cells.
**Graph 5:** Effect of treatment of selected drugs on the biochemical parameters of INH: RIF: PYZ intoxicated freshly isolated rat hepatocytes

![Graph showing biochemical parameters](image)

*Average of six independent determinations, values are mean ± S.E.M. a = P < 0.001, when compared to normal cells. y = P < 0.01, when compared to INH: RIF: PYZ treated cells. A significant increase in the levels of ASAT and ALP (P < 0.001) was observed in hepatocytes exposed to INH: RIF: PYZ when compared to normal cells. No significance difference was observed in ALAT and LDH levels when compared to the normal cells. These cells, when pretreated with the hepatoprotective drugs showed a variable results. Only few groups showed significance difference (P < 0.01) when compared to INH: RIF: PYZ treated cells.*

**DISCUSSION**

*In vitro* primary rat hepatocytes were isolated and used to study the alterations in biochemical parameters with respect to each toxicants and drugs. Important liver enzymes like ASAT, ALAT, ALP and LDH were taken into consideration. All the toxicants used in the study caused an increase in the liver enzyme levels significantly except isoniazid rifampicin and pyrazinamide which was used in combination. Hepatocytes pretreated with drugs showed significant restoration of
the altered biochemical parameters towards normal. Catechin and silymarin were found to be better in restoring the biochemical parameters back to normal. Only in case of isoniazid combination treatment, we observed variable results.

6. CONCLUSION
All the toxicants used in the study caused an increase in the liver enzyme levels significantly except isoniazid rifampicin and pyrazinamide which was used in combination. Hepatocytes pretreated with drugs showed significant restoration of the altered biochemical parameters towards normal. Catechin and silymarin were found to be better in restoring the biochemical parameters back to normal

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