A Comprehensive Review on in vitro and in vivo Models used for Hepatoprotective Activity

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Abstract
Liver is largest organ among the different organs of the body. It is the important site for metabolism and excretion. Hepatic damage affects the normal metabolic functions which may lead to severe health problems. Liver problems like cirrhosis, hepatitis and alcoholic liver diseases can be caused by continuous exposure of environmental toxins, drug abuse, alcohol abuse and over the counter drug use. Many model systems of liver failure in experimental animals are used to screen the hepatoprotective activity. In all test model systems some attempts are made to counteract this toxicosis with the substance/preparation under test. The present review paper highlights about the various in vitro and in vivo models that are prevailing for the evaluation of hepatoprotective activity of a drug.

Key Words -Hepatoprotective, in vitro, in vivo and models

INTRODUCTION
Liver is largest organ among the different organs of the body. It is the important site for metabolism and excretion [1-2]. It is also known as the “great chemical factory” of the body since it has a vital role in regulating, synthesizing, storing and secreting many important proteins, nutrients, chemicals and also in purifying clear toxins or unnecessary substances from the body. The Greek word for liver is “hepar, so medicinal terms related to liver often starts with hepato or hepatic [3-4].

The liver plays a crucial role in the maintenance, performance and regulating homeostasis of the body by involving several biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. And it act as a centre of metabolism of nutrients such as carbohydrates, proteins, lipids and excretion of waste metabolites [2, 5]. It is also an accessory digestive gland since it secret bile which plays an major role in digestion also hence maintenance of a healthy liver is necessary.

Liver problem
Commonly observed problems of liver are mentioned below:

a) Necrosis
b) Cirrhosis
c) Hepatitis- may be of viral, toxic or deficiency type.
d) Hepatic failure - Acute or chronic
e) Liver disorders due to impaired metabolic function.
   a) Disorders associated with fat (liposis) metabolism: Fatty Liver
   b) Disorders associated with bilirubin metabolism: Jaundice or which may be of different types based upon mechanisms of action and etiology.
      i. Hemolytic/Pre-hepatic jaundice.
      ii. Obstructive (post-hepatic / cholestatic jaundice)
      iii. Hepatogenous/ hepatic jaundice/cholestasis.
      (In these three conditions there occurs un conjugated per bilirubinaemia).

iv. Hereditary jaundice or pure cholestasis: Gilbert’s syndrome, Dubin Johnson syndrome and Crigler-Najjar syndrome etc, Rotor’s syndrome are some of the hereditary jaundice types.
f) Chemical/Drug induced hepatotoxicity: Generally may be hepatitis, jaundice and carcinogenesis [3, 6-7].

When hepatocytes and epithelial duct cells get injured cause accumulation of steroid in the liver which leads to further liver injury and impairment of its cellular metabolic activity and results in hepatic cell death and liver failure. This cause Activation of different cells such as natural killer (NK) cells, Kupffer cells (KC), and natural killer T (NKT) cells due to stress and that they cause injury to liver. It's been undeniable that many inflammatory cytokines, like tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-1β, formed various causes difficult in promoting tissue injury [8-9].

Occurrence of liver injury leads to cellular caspase-mediated cell death, increase tissue lipid peroxidation and depletion within the tissue GSH levels in coordination with elevated humour levels of assorted organic chemistry parameters like SGOT, SGPT, triglycerides, steroid alcohol, bilirubin, alkaline enzyme [7-9]. In patients with iron overloading, hepatotoxicity is the mostly observed since liver is the main storage site of iron [5 &10]. Hepatic damage affects the normal metabolic functions which may lead to severe health problems [1&11]. Liver problems like cirrhosis, hepatitis and alcoholic liver diseases can be caused by continuous exposure of environmental toxins, drug abuse, alcohol abuse and over the counter drug use [1, 12-13].

Sign and symptoms of liver problem.
- Jaundice, or yellowing of the skin
- Darkened urine
- Nausea
- Loss of appetite
Unusual weight loss or weight gain
Vomiting
Diarrhea
Light-colored stools
Abdominal pain in the upper right part of the stomach
Malaise, or a vague feeling of illness
Generalized itching
Varicose veins (enlarged blood vessels)
Fatigue
hypoglycemia (low blood sugar)
Low grade fever
Muscle aches and pains
Loss of sex drive
Depression

Investigation of Liver Function:
The liver function tests are performed for different purposes like accurate diagnosis of liver diseases, to determine the severity of the damage, access the prognosis and evaluate the therapy. The routinely performed liver function tests (LFTS) are as follows:

A. Abnormalities of bile pigments and bile salts excretion tests
   - Serum total direct and indirect bilirubin.
   - Urine bile salts, bile pigments and urobilinogen.

B. Serum enzymes assays
   - SGOT (AST)
   - SGPT (AST)
   - Alkaline phosphatase (ALP) and if necessary
   - γ- Glutamyl transpeptidase (γ-GT)
   - Other enzymes

C. Changes in plasma protein tests
   - Thymol turbidity test
   - Determination of total proteins, albumin globulins.

Hepatotoxins
Many tissues including liver can be injured by direct toxin (eg.CCl₄) which indirectly affects metabolic pathway of the liver (eg. galactosamine). Hepatotoxins are some chemical reagents and drugs which induce liposis, necrosis, cirrhosis, carcinogenesis and hepatobiliary dysfunctions in experimental animals. Thus liver may be affected by hepatotoxins by different ways as:
   1. Interference with hepatic bilirubin uptake, conjugation and excretion eg. Rifampicin.
   2. Dose and time dependant reactions.
      - Acute toxic hepatitis eg. Paracetamol
      - Fatty liver eg. Tetracyclin
   3. Dose independent reaction.
      - Diffuse hepatocellular damage eg. Isoniazid
      - Cholastatic hepatitis eg. Chlorpromazine
      - Granulomatous infiltration eg. Phenytoin, Chlorproamidine.

Hepatotoxicants model.
Many model systems of liver failure in experimental animals are used to screen the hepatoprotective activity. In all test model systems some attempts are made to counteract this toxicosis with the substance/preparation under test [14 & 71]. The following are some of the experimental models explained by employing some of the important hepatotoxins.
   - Carbon tetra chloride model
     1. Acute hepatic damage
     2. Chronic reversible hepatic damage
     3. Chronic irreversible hepatic damage
   - D-galactosamine model
   - Paracetamol model
   - Chloroform model
   - Ethanol model
   - Hypoxia model
   - Diclofenac model
   - Isoniazid and rifampicin model
   - Cisplatin model
   - Iron overload inducing model
   - Thioacetamide model
   - Rifampicin & isoniazid moddel [1, 15- 24].

EVALUATION OF HEPATOPROTECTIVE ACTIVITY
The extent of the protective action can be measured by estimating the enzymes and the rate of survival and can be verified histologically. The available methods are
   - In vivo methods.
   - Ex vivo methods.
   - In vitro methods.

In vivo methods.
These are of two types.
   1. Based on bile parameters: choleretic or anticholeretic activity of compounds having hepatoprotective claims are evaluated in order to ensure whether the liver disorder is due to an abnormality of bilirubin metabolism or not.
   2. Based on serum parameters: Hepatotoxicity is induced in experimental animals by the administration of known dose of hepatotoxin like carbon tetrachloride, paracetamol, D-galactosamine, thioacatamide, ethyl alcohol etc., which produce marked measurable effects, the extent of which can be measured by carrying out various liver function tests. It is very convenient laboratory method, reproducibility of results is rather poor [14&71].

1. Paracetamol-induced hepatotoxicity
Paracetamol induces acute hepatotoxicity depending upon its dosage through different routes of administration, such as
   a) Paracetamol (800mg/kg i.p.) induces centrilobular necrosis without steatosis.
   b) Paracetamol at a single dose of 3g/kg p.o. stimulates acute hepatic damage. It takes 48 hrs to induce the toxicity.

Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female rats were divided into various
groups, each group had six animals. Hepatotoxicity was using induced using paracetamol (2 g/kg b.w. p.o.) for 7 days.
On the seventh day, after 2 hrs of respective drug treatments, animals were anaesthetized using diethyl ether inhalation jar. Blood was collected through cardiac puncture and the serum was separate.

Biochemical Estimation
Liver function biochemical markers such as ALT, AST, ALP, total bilirubin and total protein have been evaluated using standard kits [25&26].

2. CCl₄ model induced
A number of CCl₄ models are devised depending upon its dosage through different routes of administration.

a) Acute hepatic damage: Acute liver damage is manifested as ischemia, hydropic degeneration and central necrosis which is caused by oral or subcutaneous administration of CCl₄ (1.25ml/kg). The maximum elevation of biochemical parameters are found to be 24 hours after the CCl₄ administration normally administered as 50% v/v solution in liquid paraffin or olive oil.

b) Chronic reversible hepatic damage: Administration of CCl₄ (1ml/kg S.C.) twice weekly for 8 weeks produces chronic, reversible liver damage.

c) Chronic, irreversible hepatic damage: Administration of CCl₄ (1ml/kg S.C.) twice weekly for 12 weeks simulates chronic, irreversible liver damage.

Experimental design
The study was performed by using Sprague Dwalley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced using CCl₄ (0.5 mL/kg, s.c.) for 9 days. On the 10th day, all the animals were sacrificed under anesthesia and blood as well as liver samples were collected for biochemical and histopathological investigation.

Biochemical investigation
Blood samples were taken by retro orbital plexus and allowed to clot at room temperature for 45 min. Centrifugation was done at 1200–1500 rpm for 20 min for separation of serum. The serum was used for the estimation of biochemical parameters namely serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) and alkaline phosphatase (ALP) by using autoanalyzer [27-30].

3. Cisplatin
Experimental design
The study was performed by using Sprague Dwalley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hapatotoxicity induced cisplatin (7.5 mg/kg i.p.) for 1st & 11th day. On the 6th, 10th and 16th day, blood sample were withdrawn from retro-orbital sinus of rats for biochemical parameters.

Biochemical assays
The collected blood samples were centrifuged to get serum. Serum urea and creatinine, ALT, AST and antioxidant activity were estimated using diagnostic kit for the assessment of liver toxicity [31-37].

4. Ethanol
Ethanol induces liposis to a different degree depending upon its dose, route and period of administration as follows:

a) A single dose of ethanol (1ml/kg) induces fatty degeneration.

b) Administration of 40%v/v ethanol (2 ml/100g/day p.o.) for 21 days produces fatty liver.

c) Administration of country made liquor (3ml/100 g/day p.o.) for 21 days produces liposis.

Experimental design
The study was performed by using Sprague Dwalley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced 40% ethanol (v/v, 2.0ml/100g body wt, p.o.) for 21 days. On 22nd day, blood was obtained from animals by puncturing retro orbital plexus. Blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 2500 rpm at 30°C for 15 mints.

Biochemical estimation
The serum markers were obtained for the estimation of various biochemical parameters including SGOT & SGPT [38-43].

5. Thioacetamide
Thioacetamide (100mg/kg s.c.) induces acute hepatic damage after 48 hrs of administration by causing sinusoidal congestion and hydropic swelling with increased mitosis.

Experimental design
The study was performed by using Sprague Dwalley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced using thioacetamide 50mg/kg, i.p. for 15 days. On 15th day after 16hr of thioacetamide, the blood samples were collected from the orbital sinus under ether anaesthesia, and liver samples of the mice were obtained after sacrificing the animal.

Biochemical estimation
The blood samples were allowed to coagulate for 10 min, and the serum was separated by centrifugation at 3500 rpm at 4°C. The liver was immediately separated, washed with cold saline and stored at 8°C. Then estimate the liver markers ALT, AST, ALP and ALB and the amount of TP and TP. [44&45].

6. Hypoxia
Experimental design
The study was performed by using Sprague Dwalley/Wistar albino male & female mice were divided into various groups, each group had six animals. Negative control group was not kept inside an hypoxic chamber. The other groups
were kept in hypoxia chamber at a concentration of O₂ 10 % and N₂ 90 % for a week. The blood and the liver were taken after 7 days of treatment to measure the malondialdehyde (MDA) levels using thiobarbituric acid (TBA) method [46&47].

7. D-galactosamine
Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced using D-galactosamide 400mg/kg, b.wt, i.p. for 7 days.
On the 8th day After 24 h of intoxication blood was withdrawn and collected in sterile centrifuge tubes and allowed to clot. Biochemical estimation
Serum was separated and used for the estimation of ASAT, ALAT, ALP, triglycerides, total proteins, albumin, total bilirubin and LDH using kits [48- 50].

8. Diclofenac
Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced using diclofenac50mg/kg, i.p. for 7 days.
After the 7th day of the experiment the rats were anesthetized, blood sample collected from the inner canthus. Biochemical estimation
Which were performed for the liver function tests (LFTs): Alanine Amino Transferase (ALT), Gamma Glutamyle Transferase (GGT), Aspartate Amino Transferase (AST). and cholesterol were estimated [51- 55].

9. Chloroform
Chloroform by inhalation or by subcutaneous administration induced hepatotoxicity with immense central necrosis, fatty metamorphosis, hepatic cell degeneration and necrosis. Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity induced chloroform 0.2 ml/kg, p.o. twice per week for 8 weeks.
At the end of the experiment, all the mice were sacrificed, and their livers and blood were collected immediately. Liver weights were subsequently measured. Biochemical estimation
The serum was isolated and stored at -80°C until analysis. According to the manufacturer’s protocols Serum markers such as AST and ALT levels were estimated using commercial kits [56- 59].

10. Iron over load
Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female mice were divided into various groups, each group had six animals. Hepatotoxicity was induced using 100 mg/kg b.w. each of iron dextran saline (i.p) on alternative days for 10 days. After the 21st day mice were fasted over night and anesthetized with ethyl ether then blood was collected by cardiac puncture. After the clotting of blood samples, sera were separated using cooling centrifuge and store at −80°C until analysis. The liver was dissected out and rinsed with ice-cold saline to eliminate the blood cells; half of them were cut, weighed, and homogenized in 10 volume of 0.1M phosphate buffer (pH 7.4) containing 5mM EDTA and 0.15M NaCl, and centrifuged at 8000 g for 30 min at 4°C. The supernatant was collected and used for the assay of enzyme activities, protein oxidation, levels of hydroxyproline content, and lipid peroxidation products. A standard graph of blood sample was prepared to estimate the protein concentration in the homogenate by Lowry method. The other half of the liver samples were weighed and digested with equi volume (1:1) mixture of sulphuric acid and nitric acid and their iron content were analysed by colorimetric method. Biochemical estimation
The blood serum was measured for Alanine amino transferase (ALAT), naspartate amino transferase (ASAT), and bilirubin, serum alkaline phosphatase (ALP) was estimated [1, 42&60].

11. Isoniazid and Rifampicin induced hepatotoxicity
Experimental design
The study was performed by using Sprague Dwaley/Wistar albino male & female rats were divided into various groups, each group had six animals. Hepatotoxicity was induced using isoniazid and rifampicin at the dose of 100 mg/kg b.w., p.o. to the experimental animals for 21 days. On 21st day blood samples were withdrawn the retroorbital venous plexus of rats without any coagulant for the separation of serum. After collecting the blood in Eppendorftubes kept aside for 1 h at room temperature and then serum was isolated by centrifugation at 2000 rpm for 15 min and stored until analyzed for various biochemical parameters. Bio-chemical studies
Serum was separated to study the biochemical parameters like SGOT, SGPT, ALP, Total Bilirubin, Direct Bilirubin, Total Protein and Total cholesterol [61- 65].

EX VIVO METHODS:
• In this method hepatocytes are isolated and the percentage of viable cells and biochemical parameters are determined as liver function tests after completion of preselected in vivo test protocol.
• These methods are somewhat better correlated to clinical models than in vitro or in vivo methods. [71].

IN VITRO METHODS:
1. Cell viability test
2. Toxicity study
3. Cell line technique
In vivo cell viability test

Overnight fasted male Sprague-Dawley/Wistar albino male & females rats were weighed, anaesthetized by using ketamine and 1% of sod.citrate was injected (i.p.) to prevent blood clotting during anesthesia by using ketamine. Rat was sacrificed by cervical dislocation and Liver lobes were isolated after cardiac and liver perfusion with Ca²⁺-Mg²⁺ free Hanks buffer salt solution (pH 7.4) for 15 min. Then hepatocytes are isolated by using in-situ under aseptic condition and placed in chilled HEPES (N-2-hydroxyethylpipерazinе-N-2-ethanesulfonic acid). These isolated hepatocytes incubated in the medium with toxins like CCl₄, thioacetamide, ethanol and paracetamol etc at 37°C for 3 h. Hepatoprotective activity was assessed by checking the viability of the cells after 3 hr of incubation using Trypan blue dye and by measuring release of cytosolic enzymes like GPT, GOT and LDH in the medium using semi autoanalyser [66].

In vitro toxicity study

In vitro toxicity studies liver slice culture system is used. The LDH release from this system was used to market to study hepatotoxicity of the hepatotoxicant. Liver slices culture were divided various sets. These sets incubated at 2 hrs with different treatment. After completion of incubation period, % of LDH release was calculated [67].

In vitro cell line study

The trypsinized single thickened cell culture and the cell count using medium containing 10% new born calf serum was adjusted to 1.0 x 10⁵ cells/ml. 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added to each well of the 96 well microtiter. After 24 hours, limited monolayer was formed, the supernatant was flicked off, washed the monolayer once and 100 μl of different drug concentrations was added to the cells in microtitre plate. Then incubated at 37°C for 3 days in 5% CO₂ atmosphere and microscopic exploration was carried out and record the observations in each 24 hours. After 72 hours, the drug solutions in the wells were replaced with 50 μl of MTT to each well and gently shaken and keep incubated for 3 hours at 37°C in 5% CO₂ atmosphere. Disacard supernatant layer and 50 μl of propanol was added and the plates were gently shaken to solubilise the formazan. Measured the absorbance using a microplate reader at a wavelength of 540 nm [68- 70].

Conclusion

The current updated report covers the various in vitro and in vivo models that are prevailing for the researchers who are involved in the research and development of a new hepatoprotective agent. This comprehensive review on the various models highlights about various toxicants that can be employed for creating hepatotoxicity with the subsequent parameters to be evaluated for the hepatoprotective effect. This updated review on the various models will be much more helpful for all those researchers who are all carrying out their investigations and research on hepatoprotective activity.

References

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