INTERNATIONAL JOURNAL OF ADVANCES IN PHARMACY MEDICINE AND BIOALLIED SCIENCES
An International, Multi-Disciplinary, Peer-Reviewed, Open Access, Indexed, Triannually Published Journal
|www.biomedjournal.com|
ISSN 2348-2109

# Experimental models and hepatotoxic agents used to study hepatoprotective effect of traditional drugs

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REVIEW ARTICLE

ARTICLE INFORMATION

*Article history*
Received: 1 December 2014
Revised: 10 December 2014
Accepted: 20 December 2014
Early view: 30 December 2014

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## ABSTRACT

Liver damage or disease is a worldwide health threat due to non-availability of specific drug and potentiality of modern drugs to add further damage. A large number of traditional drugs possesses hepatoprotective activity have been used in liver pathologies since centuries. So investigating these drugs for their hepatoprotective effect to make an effective medicine in the treatment of liver toxicity or dysfunction is promising. Different kinds of laboratory models have been used to assess the hepatoprotective action of these drugs. Hepatotoxic agents such as Carbon tetrachloride ($CCl_4$), Paracetamol, D-galactosamine are commonly used. Most widely, carbon tetrachloride ($CCl_4$) has been used to induce liver toxicity in rodents. Likewise chloroform, acrylamide, adriamycin, aflatoxin, thioacetamide, isoniazid, rifampicin, ethanol, pyrilizidine alkaloid, alphanapthoisothiocynate, tamoxifen, phalloidin, cadmium, lead and erythromycin have been also used to induce chemical injury in the liver. When the disease is induced in an appropriate animal, the traditional drugs can be tested for its therapeutic effect as well as its effective dose and toxicological profile. In experimental models, both in vivo and in vitro models of liver have been used. The hepatoprotective effect is evaluated by ability of the trial drug to prevent or mitigate the injury in different parameters like biochemical, histological changes and normalization of the volume of the liver. The present article explains the types, dose and mechanism of hepatotoxic agents along with experimental model used to study hepatoprotective effect of traditional drugs.

*Keywords:* Hepatotoxic agents; Hepatotoxicity models; Hepatoprotective effects; Traditional drugs; Experimental models; In Vitro in Vivo; liver damage; Traditional medicine.



## INTRODUCTION

Liver is the largest and the most important organ present in the body. It is the principle site for metabolism, secretory and excretory functions. The potential of hepatocyte to proliferate and regenerate along with its dual blood supply, distinguish liver from the other organs of the body. Recovery that proceeds after the liver injury due to toxic substances, food borne and viral infection is mainly attributed to its power of regeneration. The hepatocyte performs liver metabolic functions, such as 1) Formation and excretion of bile during bilirubin metabolism (Bilirubin Metabolism) 2) Regulation of carbohydrate homeostasis 3) Lipid synthesis and secretion of plasma lipoproteins 4) Control of cholesterol metabolism 5) Formation of urea, serum albumin, clotting factors, enzymes, and numerous other proteins 6) Metabolism or detoxification of drugs and other foreign substances etc. (Ghany and Hoofnagle, 2012; & Merckmanuals, 2014).

Virtually all lipid soluble, exogenous substances are metabolized in the liver. This function is carried out largely by hydroxylation by mixed-function oxidases, followed by conjugation. This process is responsible for most drug metabolism and is at the center of many drug

interactions. The liver has limited number of ways of responding to injury such as acute hepatitis, chronic hepatitis, fibrosis, tumors and carcinomas. In addition, there are a number of inherited metabolic and storage diseases of the liver. The remarkable ability of the liver to regenerate spares it from end-organ failure in most of these diseases (Gregory, 2000).

But when it is continuously and variedly exposed to environmental toxins, chemicals like CCl4, drugs, alcohol, infections, ischemia and autoimmune disorders, prescribed (antibiotics, chemotherapeutic agents) cum over-the-counter drugs can eventually lead to various liver insults like hepatitis, cirrhosis and alcoholic liver disease. Most liver disorders cause some degree of hepatocellular injury and necrosis, resulting in various abnormal laboratory test results and, sometimes, symptoms. Symptoms may be due to liver disease itself (e.g. jaundice due to acute hepatitis) or to complications of liver disease (e.g. acute GI bleeding due to cirrhosis and portal hypertension).

On the other hand, treatment options for common liver diseases are limited, and therapy with modern medicine may lack efficacy. The effectiveness of treatments such as those using interferon, colchicine, penicillamine and corticosteroids is consistent, carries the risk of adverse effect, and is often too costly and the incidence of side-effects profound. Hence, we are in the need of new drugs with minor side effects. Clinical studies demonstrated efficacy and safety of a number of herbal products in the treatment of liver diseases (Baghban, 2014). Traditional medicine provide significant source of hepatoprotective drugs. Mono and poly-herbal preparations of traditional medicine, have been used in various liver disorders since decades. According to an estimate, more than 700 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 100 plants are in clinical use as hepatoprotective. Therefore, many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective effect against liver damage in experimental animal model. Several authors have reported favorable results of traditional drugs either in animal or human studies.

**Types of hepatotoxic agents and their mechanism of liver damage (Hepatotoxicity)**

Chemicals which are administered to induce or cause liver injury are called hepatotoxic agents (Maity and Ahmad, 2012). Although a wide variety of industrial chemicals, solvents or therapeutic drugs can produce liver injury, it is apparent that their pharmacological effects on the liver differ in many ways (Drill, 2014). Hepatotoxic agents are generally divided into two groups. The first group includes those hepatotoxic chemical or agents which produce hepatotoxicity or liver damage when get metabolized in liver. While the other group of chemicals do not require metabolism to produce hepatotoxicity. Based on properties of hepatotoxic agents, the mechanism of Liver damage or injury has been proposed to involve two classic division of drug reactions, (1) drugs that directly affect the liver and (2) drugs that mediate an immune response and then produces liver injury (Ravishankar, 1995; Drill, 2014; Mehta, 2014).

In general, the pathophysiology of hepatotoxicity are still being explored and include both hepatocellular and extracellular mechanisms. The following are some of the mechanisms that have been described: (1) Disruption of the hepatocyte by covalent binding of the drug to intracellular proteins can cause a decrease in ATP levels. (2) Disruption followed by disassembly of actin fibrils in the hepatocyte produces blebs and rupture of the membrane. (3) Disruption of the transport protein interrupts bile flow. This prevents the excretion of bilirubin and cholestasis develops. (4) Covalent binding of a drug to the P-450 enzyme acts as an immunogen, activating cytolytic T-cell and cytokines. Activation of tumor necrosis factor-alpha receptor of Fas also leads to apoptosis of the hepatocyte. Damage of the hepatocyte, activated cytotoxic T-cells and cytokines may trigger innate immune cell subsets, including Kupffer cells (KC), natural killer (NK) cells, and NKT cells. These cells contribute to the progression of liver injury by producing proinflammatory mediators and secreting chemokines to further recruit inflammatory cells to the liver. It has been demonstrated that various inflammatory cytokines, such as tumor necrosis factor (TNF), interferon (IFN)-, and interleukin (IL)-1, produced during hepatic injury are involved in promoting tissue damage (Ahmad and Tabassum, 2012; Gao, 2009). Certain drugs disrupt mitochondrial function by inhibiting the synthesis of nicotinamide adenine dinucleotide and flavin adenine dinucleotide, resulting in decreased ATP production. This influence the blood supply, oxygen flow into liver and release of toxic metabolites in bile may cause injury to the bile duct epithelium (Ahmad and Tabassum, 2012; Drill, 2009; Mehta, 2014; Gao, 2009). The most common hepatotoxic agents used to induce liver injury are given below,

***Carbon tetrachloride ($CCl_4$)-induced hepatotoxicity***

The carbon tetrachloride ($CCl_4$) was previously used as grain fumigant, dry cleaning fluid, fire extinguisher fluid and for degreasing the metals. The $CCl_4$ is used as hepatotoxic agent for inducing both acute and chronic liver failure. The injury caused by carbon tetrachloride (CCl4) can be attributed to a number of mechanisms (Singh, 2012). The formation and release of free radicals and lipid peroxides is one of the mechanisms of injury. $CCl_4$ is metabolized to $CCl_3$ (trichloromethyl radicle) by specific ferrous cytochrome P-450. $CCl_3O$- is a highly reactive oxidative free radical produces peroxidation of polyunsaturated fatty acids in the endoplasmic reticulum (lipid peroxidation) (Ahmad and Tabassum, 2012; Ravishankar, 1995; Dalton, 2009; Singh, 2012). The free radicle also contains the ability to covalently bind macromolecules in the cell which inhibits proteins synthesis and produces intracellular calcium imbalances (Dalton, 2009; Manibusan, 2008; Weber, 2003). Single dose of CCl4 in rat produces centrilobular necrosis and fatty changes within 24 hours. The concentration reaches

its maximum within 3 hours of administration. No $CCl_4$ left in the liver after 24 hr. The development of necrosis is associated with leakage of hepatic enzymes into serum. Dose of $CCl_4$: 1 mL/kg body weight, i.p., 1:1 ratio ofmixture of $CCl_4$ and olive oil twice weekly for more than 8 weeks. Single dose of $CCl_4$ to induce the acute hepatotoxicity is 2.5 mg/ kg of body weight 1:1 with liquid paraffin i.p. (Dalton, 2009; Singh, 2012).

***Paracetamol-induced hepatotoxicity***

The drug is used as antipyretic and analgesic, and can cause acute liver damage in higher doses. Paracetamol administration causes necrosis of the centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm followed by large excessive hepatic lesion (Singh, 2012; Manibusan, 2008). The liver injury induced by paracetamol is mediated by its toxic reactive metabolites, N-acetyl p-benzoquineimine, formed from the parent compound by conversion through mixed function oxidases system of hepatocyte. The metabolite is detoxified by binding to glutathione. When the metabolite is formed in excessive amount, utilization of liver glutathione increases leads to its depletion in the liver. The unconverted metabolite then binds to nucleophilc macromolecules of the hepatocytes. This binding is believed to cause hepatocyte necrosis. Any condition that causes depletion of liver glutathione level will potentiate the toxicity of paracetamol.

Dose of Paracetamol: single dose of 2 g/kg P.O adminstered on the 5thday of experiment. Single dose of 3 mg/ kg on 3rd day of experiment (Singh, 2012; Eesha, 2011).

***Thioacetamide-induced hepatotoxicity***

Thioacetamide has been used as an organic solvent in the leather, textile and paper industries and as a stabilizer of motor fuels. It is a prototypical thioamide. Thioacetamide produces hepatotoxicity within a short period of time. Thioacetamide inhibit oxidative phosphorylation of the liver by causing uncontrolled entry of Ca++ ions into the hepatocytes and impairing its oxidative metabolism. It interfere the movement of the RNA leading to increase RNA content which may cause membrane injury. A metabolite of thioacetamide, thioacetamide s-diooxide is responsible for hepatotoxicity. Thioacetamide s-diooxide is highly reactive, reduces the number of viable hepatocytes as well as rate of oxygen consumption. It also decreases the volume of bile and its content i.e. bile salts, cholic acid and deoxycholic acid. I.P. dose of thioacetamide: 200 mg/kg, thrice weekly for 8 weeks induces hepatotoxicity (Ahmad and Tabassum, 2012; Ravishankar, 1995; Singh, 2012; Amin, 2011).

***D-Galactosamine-induced hepatotoxicity***

D-Galactosamine produces diffuse echopattern of hepatotoxicity mimics injury caused by acute viral hepatitis or self-limiting hepatitis with necrosis.D-Galactosamine (D-Gal) is supposed to produce hepatic injury by depleting the uridine content of the liver. The depletion leads to decreased formation of uracil nucleotide dependent synthesis of macromolecules such as RNA, protein synthesis and ultimately alter hepatocellular functions. Hepatic injury is induced by intraperitoneal single dose injection of D-galactosamine (800 mg/kg) (Ilaiyaraja, 2011).

***Ethanol/alcohol-induced hepatotoxicity***

Liver is one of the organ, most of the most susceptible to the toxic effects of ethanol. Alcoholic liver diseases (ALD) constitute alcoholic fatty liver disease, alcoholic hepatitis, and ultimately cirrhosis (Gramenzi, 2006). The plausible mechanism of liver injury was supposed to be the increase in the lipid peroxidation causes which causes changes in phospholipid composition of the membrane. Excessive production of oxy-free radicle occurs during oxidation of ethanol in the liver. The peroxidation of membrane lipids, results in loss of membrane structure and integrity. These results in elevated levels of glutamyl transpeptidase, a membrane bound enzyme in serum. Ethanol inhibits glutathione peroxidase, decrease the activity of catalase, superoxide dismutase, along with increase in levels of glutathione in liver (Gao, 2011). The decrease in activity of antioxidant enzymes superoxide dismutase, glutathione peroxidase are speculated to be the damaging effects of free radicals produced following ethanol exposure or could be due to a direct effect of acetaldehyde, formed by oxidation of ethanol. Continuous administration of ethanol- 7.9 g/kg body weight/d) for a period of 6 weeks induces liver damage in rats (Ilaiyaraja, 2011).

***Antitubercular-induced hepatotoxicity***

Antitubercular regimen containing rifampicin, Pyrizinamide and Isoniazid (INH) induced hepatotoxicity is potentially serious adverse effect. Isoniazid produces both toxic and idiosyncratic hepatic injury (Ravishankar, 1995). When given in combination, the hepatotoxic effect get enhanced (Singh, 2012). Hepatotoxicity is also enhanced by rifampicin and alcohol (Ravishankar, 1995). INH is metabolized to monoacetyl hydrazine, which is further metabolized to a toxic product by cytochrome P450 leading to hepatotoxicity. Patients on concurrent rifampicin therapy have an increased incidence of hepatitis (Grant, 2012). This has been postulated that rifampsin activates cytochrome P450 enzyme, causing an increased production of the toxic metabolites from acetyl hydrazine (AcHz). Rifampicin also increases the metabolism of INH to isonicotinic acid and hydrazine, both of which are hepatotoxic. The plasma half-life of AcHz (metabolite of INH) is shortened by rifampicin and AcHz is quickly converted to its active metabolites by increasing the oxidative elimination rate of AcHz, which is related to the higher incidence of liver necrosis caused by INH and rifampicin in combination. Rifampicin induces hydrolysis pathway of INH metabolism into the hepatotoxic metabolite hydrazine. Pharmacokinetic interactions exist between rifampicin and pyrazinamide in tuberculosis patients, when these drugs are administered concomitantly. Pyrazinamide decrease the

blood level of rifampicin by decreasing its bioavailability and increasing its clearance. Pyrazinamide, in combination with INH and rifampicin, appears to be associated with an increased incidence of hepatotoxicity (Singh, 2012; Padma, 1998; Rao, 2012).

***Acryl amide-induced hepatotoxicity***

Acrylamide (AA) is a water-soluble vinyl monomerused in the production and synthesis of polyacrylamides. AA as a water-soluble polymers used as additives for water treatment, enhanced oil recovery, flocculants, paper making aids, thickeners, soil conditioning agents, sewage and waste treatment, ore processing and permanent-press fabrics (Paulsson et al., 2001; Friedman, 2003). On the other hand, AA is a component of tobacco smoke, which is formed by heating of biological material. Therefore, smoking could potentially be a source of AA in indoor air.Monomeric AA has been shown to cause diverse toxic effects in experimental animals. Acrylamide is carcinogenic to laboratory rodents and is described by the International Agency for Research of Cancer as a probable carcinogen to humans. In the human body, AA is oxidized to the epoxide glycidamide (2, 3-epoxypropionamide) via an enzymatic reaction involving cytochrome P4502E1. AA undergoes biotransformation by conjugation with glutathione and is probably being the major route of detoxification. Daily dose of 6 mg/kg, ip for 15 d produces hepatotoxicity in female Sprague-Dawley rats (Maity, 2012).

***Pyrrolizidine alkaloid (e.g monocrotaline)-induced hepatotoxicity***

This group of alkaloids is reported to cause veno-occlusive type of liver injury. Toxic changes observed are obliteration of sinusoidal space and hepatocellular injury. Hepatic radical shows suboptimal edema and progressive fibrosis which may proceed to complete occlusion.

***Mercury-induced hepatotoxicity***

Mercury is a hazardous environmental and industrial pollutant which induces severe alterations in the body tissues of both humans and animals. The toxicity of mercury depends on the forms of the mercury compounds (elemental, inorganic and organic). Mercury can gradually accumulate in the central nervous system and kidney, thus causing damage to these organs. Hence, there have been several molecular mechanistic studies on neurological and renal toxicities induced by mercury. However, despite of the fact that the extensive biliary-hepatic cycling of mercury and of some evidence suggesting that liver plays a role in renal tubular uptake of mercury, little is known with regard to the mechanism of mercury-induced hepatotoxicity (Ung, 2010). Mercury is a transition metal and binding of mercuric ions to sulfhydryl groups ROS may cause decreased glutathione levels, leading to increases in levels of reactive oxygen species (ROS) such as superoxide anion radicals, hydrogen peroxide. These ROS enhance the peroxides and hydroxyl radicals (Oda and El-Ashmawy, 2012). These lipid peroxides and hydroxyl radical cause cell membrane damage and thus destroy the cell. Mercury also inhibits the activities of free radical quenching enzyme such as catalase, superoxide dismutase and glutathione peroxidase. Mercury causes cell membrane damage like lipid peroxidation which leads to the imbalance between synthesis and degradation of enzyme protein. The excess production of ROS by mercury may be explained by its ability to produce alteration in mitochondria by blocking the permeability transition pore. Dose of mercuric chloride: 5mg/kg body weight, through intra peritoneal injection for 20 days. Mercuric chloride dose: 2mg/kg body weight, administrated orally for 30 days (Singh, 2012; Jagadeesan, 2007).

***Erythromycin-induced hepatotoxicity***

Erythromycin estolate is a potent macrolide antibiotic, generates free radicals and has been reported to induce livertoxicity. Erythromycin when given as erythromycin stearate (100 mg/kg body weight for 14 d) or erythromycin esolate (800 mg/kg/d for 15 d) to albino rats produces hepatotoxicity in them (Ahmad and Tabassum, 2012).

***Tamoxifen-induced hepatotoxicity***

Tamoxifen (TAM), a triphenylethylene derivative, is a selective estrogen receptor modulator (SERM) (Singh, 2007) that has become the treatment of choice for women diagnosed with all stages of hormone-responsive breast cancer. It was suggested that TAM is initially metabolized in the liver with subsequent accumulation of some metabolites such as 4-hydroxytamoxifen, 4-hydroxy-N-desmethyltamoxifen and N-desdimethyltamoxifen in various tissues (Al-Jassabi, 2011). In high dose, it is a known liver carcinogen in rats, due to oxygen radical overproduction and lipid peroxidation via formation of lipid peroxy radicals. An ip dose of 45 mg/kg/d of tamoxifen citrate in 0.1 mL dimethylsulfoxide and normal saline for 6 d induce hepatotoxicity in rats (Nava, 2000).

***Adriamycin-induced hepatotoxicity***

Adriamycin (doxorubicin/ DXR) is an anthracycline glycoside antibiotic that acquires an effective and broad spectrum antitumor activity against a variety of human solid tumors like ovarian, breast, lung, uterine and cervical cancers, Hodgkin's disease, soft tissue and primary bone sarcomas, as well against several other cancer types and hematological malignancies. It is used as chemotherapeutic agent. However, it does not discriminate between a cancer and normal cell. The probable mechanism of organ toxicity is postulated to be the oxidative injury to membrane lipids and other cellular components with production of hydroxyl radicals, hydrogen peroxide and superoxide anions. Ultimately generation of superoxide anion and hydroxyl radicals cause lipid peroxidation (Summya, 2013). However, its clinical potential is contraindicated due to severe cytotoxic side effects. Based on in vitro model of toxicity using isolated hepatocytes and liver microsomes, adriamycin has been shown to undergo redox cycling

between semiquinone and quinone radicals during its oxidative metabolism. A single dose of 10 mg/kg body weight (Ahmad and Tabassum, 2012).

***Microcystin-induced hepatotoxicity***

Microcystin-LR, a cyclic heptapeptide synthesized by the blue-green algae, Microcystis aeruginosa, is a potent hepatotoxin. Microcystins are synthesized non-ribosomally by the thiotemplate functions of large multifunctional enzyme complexes. Mice and rats that were given microcystin,showed severe, diffuse, centrilobular hepatocellular necrosis, swelling and hemorrhage on histopathological examination of liver. Oxidative stress is supposed to be the mechanism of injury. Oxidative injury occurs either due to overproduction of ROS or to decrease of the cellular antioxidant levels. Lipid peroxidation and DNA damage followed as serious adverse effects of oxidative stress. In mice sub lethal doses of microcystin (20 µg/kg) for 28 weeks (Singh, 2012; Lovell, 1989; Ding, 2003).

***Lead-induced hepatotoxicity***

Lead is a blue gray, divalent matter and highly toxic, present naturally in the earth crust spread in the environment through activities. The mechanism of lead induced hepatotoxicity is occurring by lipid peroxidation and generation of reactive oxygen species (Gurer, 2000). Exposure to even low levels of lead may have potential hazardous effects on brain, liver, kidneys and testes. Autopsy studies of lead-exposed humans indicate that among soft tissue, liver is the largest repository (33%) of lead, followed by kidney (Ahmad and Tabassum, 2012). Lead induced hepatic damage is mostly occurring through lipid peroxidation (LPO) and disturbance of the pro-oxidant antioxidant balance by generation of reactive oxygen species (ROS). Lead toxicity lead to free radical damage by two separate pathway: (1) generation of ROS, including hydroperoxides, singlet oxygen, and hydrogen peroxide and (2) the direct depletion of antioxidant reserves. Dose: lead nitrate; 50mg/kg body weight daily orally for 40 days (Singh, 2012).

**Types of Experimental models of Liver**

The experimental models of liver used for evaluation of hepatoprotective effect of traditional drugs can be grouped under:

In vitro models (primary cultured hepatocytes for primary screening),
In vivo models (experimental hepatocellular injury),
Models representing hepatic encephalopathy
Models representing obstructive jaundice,
Models representing jaundice induced by hemolytic anemia,
Models for inducing allergic hepatitis,
Models involving injury to kupffer cells,
Models for assessing hepatic regeneration,
Miscellaneous (Ravishankar, 1995).

The commonly used models are mentioned below:

**In vitro model**

According to the method of Seglen et al. (2012) the procedure involves isolating liver under aseptic condition and placing in HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid) buffer containing HEPES (0.01 mol/L), NaCl (0.142 mol/L) and KCl (0.0067 mol/L), pH 7.4 (Pradeep, 2009; Seglen, 2012). The liver are then cut into small pieces and incubated with a second buffer containing HEPES (0.1 mol/L), NaCl (0.0667 mol/L), KCl (0.0067 mol/L) and 0.5% Collagenase type Ⅳ, pH 7.6, for about 45 min at 37℃ in an incubator with constant shaking. Hepatocytes ere obtained after filtration and cold centrifugation (4℃, 200 rpm for 2 min, and three times) and suspended in HEPES buffer Ⅰ. The viability of the hepatocytes was assessed by trypan blue (0.2%) exclusion method (Seglen, 2012). Fresh hepatocyte preparations and primary cultured hepatocytes are cultured to study the hepatoprotective effect of drugs. Hepatocytes are treated with hepatotoxic agents and the efficacy of the test drug is evaluated. Hepatoprotective activity of test drug is determined by measuring an increase in the percentage of viable cells in that group of cells incubated with extracts, compared with the control and toxicant-alone groups. Reversal of toxin-induced elevations in the level of enzymes is also considered to assess hepatoprotective activity (Pradeep, 2009). The activities of the transaminases released into the medium are determined. An augmented activity of marker transaminases in the medium indicates liver damage. Parameters such as hepatocytes multiplication, morphology, macromolecular synthesis and oxygen consumption are determined (Pradeep, 2009; Ahmad and Tabassum, 2012). Thus in vitro model provide advantages like (1) smaller quantity of test drug will be required than in vivo models (Ravishankar, 1995) (2) more directly assess product performance, than do conventional human pharmacokinetic BE (bioequivalence) studies, since *in vitro* studies focus on comparative drug absorption from the two products (Polli, 2008) (3) reduce costs and (4) reproducibility is better, variation in the obtained results are found very less (Ravishankar, 1995).

**In Vivo Models**

To induce liver damage in experimental animals, a toxic dose or repeated dose of a hepatotoxic agent is administered. Carbon tetrachloride ($CCl_4$) induced liver damage continues to be the most frequently employed method. Oral, subcutaneous, intramuscular and intraperitoneal routes have been employed. Liver of mice and rat are mostly used as experimental model. An augmented level of liver enzymes such as glutamate pyruvate transaminase (GPT), glutamate oxaloacetate transaminase (GOT) and alkaline phosphatase in the serum indicates liver damage (Ahmad and Tabassum, 2012; Ravishankar, 1995; Amat, 2010). The Traditional test drug is administered along with or prior to and/or after the toxin treatment. If mortality was observed in two out of three animals, then the dose administered is considered as toxic dose of traditional test drug.

However, if the mortality is observed in only one animal out of three animals then the same dose was repeated again to confirm the toxic effect. If no mortality is observed, then higher doses can be employed for further toxicity studies (Pradeep, 2009).

**Parameters for evaluation of hepatoprotective effect of traditional drugs**

Depending upon type of test drug effect intended, variation in the parameters are observed. Liver damage and recovery from damage are assessed with following markers,

***Morphological:*** Based on macroscopic features, weight (organ/ body weight ratio) and sometimes volume of the liver.

***Biochemical:*** *B*y quantifying serum marker enzymes, bilirubin, and bile flow (Amat, 2010). Activities of AST and ALT in the serum can be assessed spectrophotometrically using a commercially available kit (Dalton, 2009). The biochemical parameters used for assessment of hepatoprotective activity are given in the table 1 (Ravishankar, 1995).

***Histopathological changes:*** Routine sectioning followed by staining with haematoxylin and eosin of the derived tissue (Pradeep, 2009; Ravishankar, 1995; Ahmad and Tabassum, 2012; Amat, 2010) followed by light microscopy. The presence of apoptotic hepatocytes can be determined qualitatively using the DeadendTM colorimetric TUNEL system (Promega, Madison, WI). An average of 3000 total cells per animal can be counted in order to determine the percent of apoptotic hepatocytes (Dalton, 2009).

***Pharmacodynamics:*** Effect on barbiturate sleeping time (hexobarbitone 60 mg kg $^{-1}$ or pentobarbitone 30 mg kg-$^{1}$- i.p). Therapeutic efficacy of a drug against diverse hepatotoxic agents differs especially when their mechanism of action vary. Consequently, the efficacy of each drug has to be tested against hepatotoxic agents which act by varied methods (Pradeep, 2009; Ahmad and Tabassum, 2012; Ravishankar, 1995; Amat, 2010).

**Table 1**. Biochemical parameters for assessment of liver damage by hepatotoxic agents in the serum.

| Enzymatic parameters | Non enzyme parameters |
|---|---|
| Transaminases (GOT & GPT) | Bilirubin, Total protein and Total lipids |
| Alkaline & Acid phosphatases | Total & Free cholesterol |
| Lactic dehydrogenases | Triglycerides and Free fatty acids |
| Dehydrogenase | Globulin and Phospholipids |
| S-Glutathione transferase | Plasma Lactate, Ceruplasmin |
| Succinic dehydrogenase | Orosomucoid |
| Glutamate dehydrogenase | BSP Clearance |
| Plasma lecithin cholesterol acyl transferase | Lipoprotein X, A/G Ratio |

**Animal models of hepatic encephalopathy (HE) in acute liver failure (ALF)**

Animal species such as large animals (dogs, goats, pigs, rabbits) and rodents (rats, mice) have been used for the study of HE. Large animals have the advantages of facilitating repeated sampling of body fluids, biopsies, the use of techniques such as visual/auditory evoked potentials and electroencephalography (EEG).But recent studies showed that rat and mouse are the most widely used species for the study in HE. Hepatotoxic agents used to induce ALF include galactosamine, acetaminophen, thioacetamide and azoxymethane. Other toxins such as phosphorus, nitrosamines and $CCl_4$ are known to produce ALF but HE resulting from these later toxins has not been well characterized. The $CCl_4$ model is useful in demonstrating astrocytic response at the level of RNA synthesis. Galactosamine is directly hepatotoxic while acetaminophen and thioacetamide are metabolized in the liver via the microsomol P-450 system. Bridging necrosis and a lymphocytic inflammatory infiltrate are the pathological changes caused by these toxins in the liver (Butterworth, 2009).

**Models for obstructive jaundice**

Jaundice due to obstruction in bile flow is associated with complication particularly infections related to defective host immune response whereas other are systemic. Biliary fibrosis and cirrhosis occurs when there is repeated injury or prolonged obstruction of either the intrahepatic or interhepatic biliary system. Mechanism of live damage associated with liver associated with obstructive jaundice is complex and multifactorial. Hepatotoxicity due to bile acids is one of the underlying cause for the pathogenesis of the disease. Cholestasis induce an inflammatory response in the liver, although the mechanism is still not known. Several etiological factors have been identified as the causative factors such as gall stones, postoperative strictures, and infective cholangitis etc. Studies have been proved that intense oxidative stress and lipid peroxidation in plasma and liver tissue in animals produces cholestasis (Ravishanker, 2009; Serdar, 2014).

*In extrahepatic obstructive hepatitis*: after ligating the CBD (common bile duct) of rat under anesthesia, animal is allowed to recover. Then test drug should be administered following the suitable dose schedule and sacrificed on 8$^{th}$ day. Estimation of liver biochemistry, plasma total lipid, phospholipids, triglycerides, free and total cholesterol along with free fatty acids should be done.

*In intrahepatic obstructive jaundice*: evaluation of test drug in intrahepatic obstructive jaundice is done for assessing the choleretic activity and anticholestatic activity against the cholestatis induced by hepatotoxic agents like paracetamol (600 mg kg-$^{1}$), thioacetamide (200mg kg$^{-1}$), oestradiol (5mg kg$^{-1}$) etc. Rats and rarely guinea pigs are used as animal models.

**Models for hemolytic anemia**

Rat models are mainly used. Phenyl hydrazine is used in injectable dose of 14 mg kg-1 for 5 days. On 8$^{th}$ day blood is drawn from heart through cardiac puncturing under anesthesia. Haematological and liver's biochemical parameters are evaluated.

**Models for allergic hepatitis**

Male guinea pigs and mice are most commonly used. According to Mizoguchi et al. (1991) a drug-induced drug-induced allergic hepatic disorder model was established using a hapten and carrier. Penicillin G is used to bind glycine for the preparation of N-hydroxy succinic imidylglycinyl benzylpenicillate (PG-Gly-OSu). Using this as the hapten and liver protein as the carrier, guinea pigs are sensitized. This leads to binding of liver protein to PG-Gly-OSu. After 2 weeks, the sensitized guinea pigs are directly challenged with hepatocytes bound to PG-Gly-OSu through a mesenteric vein and hepatocellular disorder is induced. The results suggest that the combination of PG-Gly-OSu as the hapten and liver protein as the carrier elicits a hepatocellular disorder similar to drug-induced allergic hepatitis (Mizoguchi, 1991). Other models such as Anti- basic liver protein (BLP) antibody induced liver injury in DBA, anti-liver specific protein (LSP) antibody induced liver injury in mice and immunologically induced liver injury in guinea pigs are commonly used (Ravishanker, 2009).

**Experimental models for kupffer cell's hepatotoxicity**

Data supports the role of kupffer cell activation by endotoxin early after D-galactosamine treatment is an important event in mechanism of hepatotoxicity in rats. (Stachlewitz, 1999) Ricin, glycoprotein in castor seed is used as a toxicant for kupffer cells Dose: 15µg $kg^{-1}$s.c/ i.p) (Ravishanker, 2009)

**Experimental models for assessing hepatic regeneration**

Liver regeneration is a complex process. Capacity of regeneration is the main factor for recovery after extensive liver insults. Regeneration models may be in vitro and in vivo. Cultured hepatocytes (in vitro model) have been very different physiological responses relative to in vivo models, and it has been increasingly recognized that non-parenchymal cells may play an important role in in vivo regeneration because their interaction with hepatocytes is implicated in all physiological responses of the liver. In 1931, Higgins and Anderson published a model of 70% hepatectomy in adult rats, which has been employed heavily in investigations of hepatic regeneration. Small animals, such as mice and rats are commonly used (Tannuri, 2007). Magalhães, et al. (2014) performed 60% hepatectomy in male rats to assess the effect of L-glutamine in liver generation through liver weight changes. Analysis of liver regeneration was made by the Kwon formula, study of liver function (AST, ALT, GGT, total bilirubin, indirect and indirect bilirubin and albumin) and analysis of cell mitosis by hematoxylin-eosin (Magalhães, et al., 2014).

## CONCLUSION

Tremendous efforts have been made to search for the molecular, cellular and pharmacological bases of traditional medicines. Research studies on Indian system of medicine such as Unani, Ayurveda, Siddha, homeopathy and other system based on natural medicine aim to identify the active ingredients of herbal drugs and investigate the mechanism of action. The models used by conventional medicine seem directly relevant. In addition, drug safety and efficacy evaluation must begin in vitro and then in animal for ethical reasons. Therefore, cell line models and animal models that have been successful in western medicine are readily available for complementary and alternative medicine (CAM) development. When the disease is induced in an appropriate animal, the traditional drugs can be tested for its therapeutic effect as well as its effective dose and toxicological profile. Moreover both in vitro and in vivo models of rats are used for assessment of heptoprotective activity of the traditional drugs. Various hepatotoxic agents including Carbon tetrachloride ($CCl_4$), Paracetamol, D-galactosamine induce hepatic injury or inflammation by mechanism of free radicle generation and lipid peroxidation. The experimental models and hepatotoxic agents in the present article will enable further elucidation of the mechanisms involved in liver injuries, as well as the development of hepatoprotective therapeutic interventions in the complex phenomenon.

## ACKNOWLEDGMENTS

We appreciate the cooperation of Mr. Shahzad Ahmad in searching databases, Librarian Mr. Bhopal and Library Assistants of Faculty of Medicine (Unani), Jamia Hamdard, New Delhi, India.

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