"ROLE OF TISSUE REPAIR IN DRUG INDUCED LIVER INJURY [DILI] MODEL OF FEMALE SWISS ALBINO MICE"

A Thesis Submitted to

NIRMA UNIVERSITY

in Partial Fulfillment for the Award of the Degree of

MASTER OF PHARMACY

IN

PHARMACOLOGY

BY

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CERTIFICATE

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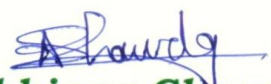**Nirma University**

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DECLARATION

I hereby declare that the dissertation entitled "Role of tissue repair in Drug induced liver injury model (DILI) of female Swiss albino mice", is based on the original work carried out by me under the guidance of Dr. Murali B., Associate professor & Head, Department of Pharmacology, Institute of Pharmacy, Nirma University and Mr. V.G.S Sharma, Manager (Business Development)Flair Labs, Surat. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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General Remarks

◆ Optical densities were recorded on SHIMADZU-1800 UV-spectrophotometer and are uncorrected.

◆ The biochemical estimations were recorded on Prietest 2.622 A, ROBONIK TOUCH Biochemistry Analyzer.

◆ Photographs of histopathological studies were taken by OLYMPUS-CX21FS1 Trinocular microscope.

LIST OF ABBREVATIONS

2-OH-CBZ	2-Hydroxy-CBZ
3-OH CBZ	3-Hydroxy-CBZ
ABCB1	ATP-binding cassette-1 Protein
ABCB11	ATP-binding cassette-11 gene
ABCB4	ATP-binding cassette-4 gene
ABCC2	ATP-binding cassette, sub-family C gene
ACA	Clavulanic Acid
ADRs	Adverse Drug Reactions
ALT	Alanine Aminotransferase
BSEP	Bile Salt Export Pump
CBZ	carbamazepine
CBZ-E	CBZ 10.11-epoxide
CSF	Cerebral Spinal Fluid
CT	CBZ Treated
CYP	Cytochrome P
DILI	Drug Induced Liver Injury
DISC	Death Inducing Signaling Complex
FasL	FAS ligand
FDA	Food and Drug Administration
GABA	Gamma amino butyric acid
GATA-3	GATA-binding domain-3
GIT	Gastro intestinal tract
HLA	Human Leukocyte Antigen
HMGB1	High-Mobility Group Box 1
IFN-ɣ	Interferon Gamma
IL	Interleukin
LDH	Lactate Dehydrogenase
M&V	The Maria and Victorino
MDH	Malate Dehydrogenase
MDR3	Multidrug resistance protein 3
MPT	Mitochondrial Permeability Transition
MRP	Multidrug Resistance-Related Protein
MRP2	Multidrug resistance protein 2
NAT2	N-acetyltransferase 2
OLTx	Orthotopic Liver Transplantation
OR	Odd Ratio
POLG	DNA Polymerase Gamma Gene
RAGE	Receptor for advanced glycation end products
ROR	Retinoid-Related Orphan Receptor
ROS	Reactive Oxygen Species
RUCAM/CIOMS	The Rousse Uclaf Causality Assessment Method of the Council of International Organization of Medical Science

S.E.M	Standard Error Of Mean
SGOT	Serum Glutamic Pyruvic Transaminase
SGPT	Serum Glutamic Pyruvic Transaminase
TLR4	Toll like receptor- 4
TNF α	Tumour Necrosis Factor α
VC	Vehicle control
VLDLs	Very Low Density Lipoproteins

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1. ABSTRACT

The liver is the central site for the biotransformation of xenobiotic chemicals and therefore is involved in the detoxifying mechanism of the body. In this process it is exposed to high concentrations of toxicants and toxic metabolites in the blood making it susceptible to injury. The liver is principle organ for the regulation protein, carbohydrate and lipid metabolism. The liver contains parenchymal cell (Hepatocyte) and non-parenchymal cell (Kupffer cell, Ito cell and endothelial cells). The hepatocyte regulates the level of the CYP's enzyme which is important for the detoxification process. Liver has a strong potential for regeneration after physical, biological or chemical injury. The stimulation of tissue repair is a biologic response that accompanies injury, quantifying this response in addition to measuring injury might be helpful in predictive toxicology. The hepatocyte regulates the proliferation as well dynamic aspect of liver. Liver injury and tissue repair are simultaneous but opposing parallel responses to administration of toxic chemicals. A wide variety of additional experimental evidence confirms the central role of stimulated tissue repair as a decisive determinant of the final outcome of liver injury inflicted by hepatotoxicants.

The drug induced injury (DILI) is more common because of the high exposure to the chemical, drug and their metabolite or even due to drug interaction. The prevalence of drug induced liver injury is about 14% of all liver failure cases. The idiosyncratic nature and poor prognosis of DILI make this type of reaction a major safety issue during drug development, as well as the most common cause for the withdrawal of drugs from the pharmaceutical market. The drug overdose leads to the liver injury and can cause a diverse array of liver injury, which may be acute or chronic. Carbamazepine is well established antiepileptic drug which is having side effect of acute hepatitis and hepatocellular injury at higher dose. The CBZ Induced liver injury model is recently studied for the mechanism of CBZ induced liver injury. But role of tissue repair (liver regeneration) is not reported till-date. Whereas other pharmacological model of DILI like Paracetamol, Thioacetamide and Carbon tetrachloride have already been studied for the role of liver regeneration. So objectives of the present study are - 1. To test weather another strain of mice (Female Swiss Albino) is also susceptible to CBZ induced

liver injury. 2. To study the role of liver regeneration (tissue repair) in CBZ induced liver injury model.

Mice were divided into two groups like-Vehicle control (VC) and the CBZ treated (CT). Vehicle control group animals were given the vehicle (corn oil) whereas the CBZ treated groups of animal were given carbamazepine (350mg/kg, p.o-4 days & 800mg/kg, p.o. on 5th day) suspended in corn oil. After the 5th day of dosing both blood and liver sample are collected at different time interval like- 0, 12, 24, 36, 48, 72, 96 hrs. Blood sample was used for biochemical analysis i.e. Serum SGOT, SGPT and Glucose level estimation and liver tissue used for liver glycogen and histopathological analysis.

The hepatic injury causes the leakage of enzyme from the cell due to altered permeability of membrane resulting in increased level in serum. The early liver injury in CBZ treated group was evident by the elevated level of the SGPT and SGOT enzyme level as compared to the Vehicle control group. The elevation of SGPT and SGOT was gradually increased up to 48 hrs time point. In contrast, no elevation of enzyme levels was observed in Vehicle control group at any time point. The elevated level of the serum SGPT and SGOT level up to 48h indicates maximum tissue injury mediated by the primary and secondary inflammation. The primary inflammation was occurred due to the CBZ and its metabolite i.e. CBZ 10, 11 epoxide and after that the secondary inflammation induced by damage hepatocyte which activate further release of inflammatory mediator. Further the enzymes level was declined at 96 hrs indicate the tissue repair response.

The gluconeogenesis and glycogenolysis were simultaneous regulated by the hepatocyte. The levels of glycogen in the hepatocyte and serum glucose indicate injury and energy profile of the hepatic tissue. The level of glucose were elevated up to 24 hrs indicate the liver tissue repair process, that demand energy leading to gradually depletion of glucose as reflected at 36 & 48 hrs time point indicating consumption of energy in the process of tissue repair.

The level of glycogen supports the glucose level changes during tissue repair and injury process. The decline was gradually observed up to the 48 hrs time point in CBZ treated group which reflect the increase in demand of energy required for the tissue repair response. The

glycogen was converted into the glucose to meet energy requirement, later glycogen levels was elevated up to 96 hrs suggested that tissue repair is almost completed that resulted in stabilization of glycogen level. The proliferation of the cell could be maximum at 36 & 48 hrs as evident from decreased glucose and glycogen levels and thereafter stabilized slowly.

The histopathological analysis showed that gradual increase in extent of necrosis as observed up to 0 - 48 hrs time points which support liver injury in CBZ treated mice. Whereas vehicle control mice show normal liver archistructure. The necrosis was maximum at 48 hrs and thereafter decline gradually indicating the completion of tissue repair response. The presence of the apoptotic bodies at 72 and 96 hrs indicates that the tissue repair was terminated at this point or gradual cessation of the proliferation of the cell. The presence of occasional mitosis activity at 36 & 48 hrs reflects tissue repair response at that time point interval. The decline in the necrosis and the reduction in the inflammation, apoptotic bodies showed after 72 hrs indicate that the tissue repair or liver regeneration has been completed at later time point. The histopathology result supports the biochemical data of CBZ induced liver injury.

In conclusion, female Swiss albino mice is susceptible to CBZ induced liver injury. This finding suggested that the tissue injury was observed due to the toxic dose of the CBZ. Whereas tissue repair response play an important role in the reversal of CBZ induced liver injury. The elevated level of liver injury markers and histopathological data suggest the liver injury severity, while the presence of the mitotic body, decline of the liver injury markers and consumption of energy substrate indicate that tissue repair has been simultaneously accompanied to regress the liver injury induced by CBZ in female Swiss albino mice.

2. INTRODUCTION

Liver is the largest organ of the body comprising 2-3% of the total adult body weight, is primarily concerned with the metabolic activity of organisms (Sheila and Dooley et al., 1993). It is also the central site for the biotransformation of xenobiotic chemicals and therefore is involved in the detoxifying mechanism of the body. Liver is responsible for detoxifying the chemical substances and in this process it is exposed to high concentrations of toxicants and toxic metabolites in the blood making it susceptible to injury (Glaister et al., 1986).

Liver is composed of different cells including hepatocytes that account about 60% of hepatic cells and remaining are endothelial cells, Kupffer cells and "Oval" cells (a population of periportal cuboidal cells with ovoid nuclei believed to represent liver stem cells). The liver receives cardiac output via the hepatic portal vein and hepatic artery. The hepatic portal vein carries the absorbed nutrients from the Gastro intestinal tract (GIT) to the liver, which takes up, stores, and distributes nutrients and vitamins. The liver plays an important role in maintaining blood glucose levels. It also regulates the circulating blood lipids by the amount of very low density lipoproteins (VLDLs) it secretes. Many of the circulating plasma proteins are synthesized by the liver. In addition, the liver takes up numerous toxic compounds and drugs from the portal circulation. It is well equipped to deal with the metabolism of drugs and toxic substances. The liver also serves as an excretory organ for bile pigments, cholesterol, and drugs. Finally, it performs important endocrine functions (Tso and McGill et al., 2002).

The liver is only organ which is having capability to regenerate. The deviation in Ratio of liver mass and body mass trigger a modulation of either hepatocyte proliferation or apoptosis, in order to maintain the liver's optimal size. The hepatocyte is regulatory bodies for maintenance of regeneration process. Hepatocyte growth factor, epidermal growth factor, transforming growth factor, cytokine, and transcriptional factor are key regulator of DNA synthesis within the cell and also the cell architecture maintenance. Co-mitogen (Insulin and Glucagon) also having important role in the hepatocyte proliferation (Court et al., 2002).

It accounts for 9.5% of all suspected adverse drug reactions (ADRs) and for a significant proportion of fatal ADRs (Lazarou et al., 1998). DILI may be a direct toxic effect or an

immunological reaction to either the drug or an active metabolite. Drugs can cause a diverse array of liver injury, which may be acute or chronic. The idiosyncratic nature and poor prognosis of DILI make this type of reaction a major safety issue during drug development, as well as the most common cause for the withdrawal of drugs from the pharmaceutical market (Temple et al., 2002).

There are no surveillance mechanisms in place to monitor adverse drug reactions, including DILI, are under-reported (Lasser et al., 2002). Controlled clinical trials provide reliable information about abnormal liver test results that are associated with specific medications, but these generally do not detect rare adverse drug reactions, So most cases of idiosyncratic hepatotoxicity are not detected (Bell et al., 2009; Larrey et al., 2002).

A key future in finding the causes of liver injury is depend upon the drug administration, dose of drug, as well as metabolic end product of drug which led to abnormal physiological symptoms, as well as the withdrawal of therapy and resolution also give idea about the diagnostic feature in liver injury. Because many drug-induced liver injuries are much more severe with repeated drug exposure, extra care has to be taken during prognosis profile. The occurrence of liver injury has to be accessed for the discriminate between idiosyncratic reaction and predictable dose dependent toxicity profile of particular drug (Au et al., 2011).

The predominant clinical presentation is acute hepatitis, hepatocellular inflammation and/or cholestasis. The pathogenesis of drug-induced liver disease usually involves the participation of the parent drug or metabolites that either directly affect the cell biochemistry or elicit an immune response. Each hepatotoxin is associated with a characteristic signature regarding the pattern of injury and latency (Kaplowitz et al., 2004).

 "Drug Induced Liver Injury is a liver injury induced by a drug or herbal medicine resulting in liver test abnormalities or liver dysfunction". The mechanism of the drug induced liver injury involves the major three pathways:

a) Direct toxicity via the metabolic product of drug which directly inhibits the cell normal pathophysiology via the activation of inflammatory mediator and transcriptional factor.

b) The second pathway in which drug undergo intrinsic pathway which led to the release of intracellular mediator which causes pro apoptic gene induction led to the mitochondrial permeability transition by formation of apoptosome causes apoptosis of cell.

c) The drug metabolites can be electrophilic chemicals or free radicals that undergo or promote a variety of chemical reactions, such as the depletion of reduced glutathione; covalently binding to proteins, lipids, or nucleic acids; or inducing lipid peroxidation. All of these have consequent direct effects on organelles such as mitochondria, the endoplasmic reticulum, the cytoskeleton, microtubules, or the nucleus (Kaplowitz et al., 2001).

The carbamazepine (CBZ) is widely used antiepileptic drug which is safe at lower dose but at higher dose it produces cutaneous as well as systemic toxicity. CBZ action appears to be mediated by a slowing of the rate of recovery of voltage-activated Na+ channels from inactivation. These effects CBZ of are evident at concentrations in the range of therapeutic drug levels in Cerebral Spinal Fluid (CSF) in humans. The effects of carbamazepine are selective at these concentrations, in that there are no effects on spontaneous activity or on responses to iontophoretically applied Gamma amino butyric acid (GABA) or glutamate. The CBZ metabolite, CBZ -10, 11-epoxy also limits sustained repetitive firing at therapeutically relevant concentrations, suggesting that this metabolite may contribute to the anti-seizure efficacy of CBZ (Goodman and Gilman's et al., 2006).

CBZ develop severe, potentially life-threatening idiosyncratic reactions such as mixed Cholestasis, agranulocytosis, hepatitis and Stevens-Johnson syndrome. The reactive metabolite of the CBZ i.e. (CBZ 2, 3- epoxide and CBZ- 3 OH) causes hepatocellular necrosis and autoimmune hepatotoxicity. The mechanism by which it causes liver injury included mitochondrial permeability transition and production of reactive oxygen species (ROS) in hepatocyte which led to the apoptosis and necrosis of cell (Pallock et al., 1987; Kaufman and Shapiro et al., 2000; Björnsson and Olsson et al., 2005).

Higuchi S et al., (2012)studied the mechanism by which Carbamazepine induces liver injury in Male Balb/c mice. They were found that CBZ 10, 11- epoxide metabolite is responsible for the CBZ Induced hepatotoxicity. CBZ is metabolized in hepatocytes by

Cytochromes P450 enzyme, the produced reactive metabolite induces ROS production in macrophages, and then danger signals released from macrophage activate Toll like receptor- 4 (TLR4) and Receptor for advanced glycation end products (RAGE). The activated TLR4 and RAGE lead to the secretion of proinflammatory cytokines and chemokine's, which result in inflammation in the liver. The necrotic hepatocytes secrete the ligands of TLR4 and RAGE, which induce further inflammation in the liver.

The CBZ Induced animal model is recently studied and liver is having fascinating aspect of regeneration after insult depends upon the chemical nature of substances. Role of tissue repair (liver regeneration) is not reported till date whereas other pharmacological model of DILI likes Paracetamol. Thioacetamide and Carbon tetrachloride have already been studied for the role of liver regeneration (Mangipudi et al., 1995). So the objectives of the present study are -

1. To test weather another strain of mice (Female Swiss Albino) is also susceptible to CBZ induced liver injury.

2. To study the role of liver regeneration (tissue repair) in CBZ induced liver injury model.

3. REVIEW OF LITERATURE

3.1 EPIDEMIOLOGY

Drugs can cause a diverse array of liver injury, which may be acute or chronic. Drug-induced liver toxicity is a common cause of liver injury. It accounts for approximately one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver disease (Kaplowitz, 2001). Drug-induced liver injury (DILI) is a significant cause of morbidity and mortality accounting for at least 13% of acute liver failure cases in the US (Ostapowiczet al., 2002).

Drug-induced liver injury (DILI) accounts for 9.5% of all suspected adverse drug reactions (ADRs) (Lazarou et al., 1998). Pre-clinical drug trials detect common DILI, but many are rare (1 in 10,000 to 100,000) and detected only during post-marketing surveillance. DILI is the most common reason for discontinuation of a new drug's development and withdrawal of an established one (Temple et al., 2002). Over a thousand drugs have been associated with DILI. DILI accounts for 14% of all cases of acute liver failure and is the leading cause for patients to be listed for super-urgent orthotopic liver transplantation (OLTx), largely because of Paracetamol toxicity (Chalasani et al 2008). DILI cases have been reported to constitute approximately 6% of all out-patients and 3% of referrals and to occur more often in women (De Valle et al., 2006). The incidence of drug-induced hepatitis is higher in patients over 40 years of age (Marti., 2005).

The drug hepatotoxicity accounted for 4% of all cases of new-onset jaundice, but most cases of drug hepatotoxicity (24 patients) were attributable to acetaminophen toxicity and idiosyncratic DILI occurred in only 5 patients (0.7% of total study population). During the last decade, drug-induced liver injury has led to the withdrawal of a number of drugs from the market (Black et al., 1975; Bakke et al., 1995; Zimmerman et al., 1999; Zimmerman et al., 2000; Senior et al., 2003). Among US prescription medicines, daily doses of oral medications were associated significantly with liver failure, liver transplantation, and death from DILI by hepatocellular, cholestasis and mixed cholestasis pathological condition. In a study of approximately 600 DILI cases, only 9% of patients received less than 10 mg/day of

medication, whereas 14% received 11–49 mg/day and 77% received more than 50 mg/day. In the Spanish Hepatotoxicity Registry, 77% of patients with DILI received medications with daily doses greater than 50 mg (Bjornsson et al., 2008).

3.2 TYPE OF DRUG INDUCED LIVER INJURY

The drug induced liver injury is divided into two main types. The liver injury from hepatotoxins results from either dose dependent or dose independent action. The dose dependent actions are mainly caused due to drug overdose. The dose independent reactions are due to the idiosyncratic reaction.

3.2.1 Dose Dependent Liver Injury

Drugs or toxins that show hepatotoxicity have predictable dose-response curves (higher concentrations cause more liver damage) and well characterized mechanisms of toxicity, such as directly damaging liver tissue or blocking a metabolic process. As in the case of acetaminophen overdose, this type of injury occurs shortly after some threshold for toxicity is reached (Davies et al., 1985). The acetaminophen produces liver injury through to the excessive acetaminophen overwhelms the normal conjugation pathways of sulfation or glucuronidation, mandating that acetaminophen is metabolized by the alternate pathways through cytochrome P450. N-Acetylp- benzoquinoneimine is produced, which is highly reactive and depletes glutathione. Accumulation of N acetyl- p-benzoqinoneimine leads to cell death and hence hepatocellular necrosis.

3.2.2 Dose Independent Liver Injury/ Idiosyncratic Reaction

Idiosyncratic injury occurs without warning, when agents cause non-predictable hepatotoxicity in susceptible individuals which is not related to dose and has a variable latency period (Zimmerman et al., 1978). This type of injury does not have a clear dose-response or temporal relationship, and most often does not have predictive models. In adverse drug reactions involving overdoses, the toxic effect is simply an extension of the

pharmacological effect (Type A adverse drug reactions). On the other hand, clinical symptoms of idiosyncratic drug reactions (Type B adverse drug reactions) are different from the pharmacological effect of the drug (Davies et al., 2003). Carbamazepine, Ketoconazole, Troglitazone, Trovafloxacin and Thioacetamide causes this type of reaction include hepatocellular as well as autoimmune mediated hepatotoxicity.

3.3 MECHANISM OF DRUG - INDUCED LIVER INJURY

The liver is often involved in drug toxicity due to its significant role in drug metabolism (Grattagliano et al., 2005). DILI is a multistep process that involves both direct drug injury and the subsequent activation of inflammatory pathways by the stimulation of intrinsic and extrinsic pathway. The occurrence of a particular liver injury depends upon the environmental condition and individual's genetic susceptibility establishes the milieu for the development of cellular and host injury (Kaplowitz, 2005). The initial steps of injury are triggered by the drug, or more commonly, drug metabolites.

Although the exact mechanism of DILI remains largely unknown, it appears to involve two pathways- cellular mediated hepatotoxicity and immune mediated reactions. In most instances, DILI is initiated by the bioactivation of drugs to chemically reactive metabolites, which have the ability to interact with cellular macromolecules such as proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress. Additionally, these reactive metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. This impairment of cellular function can led to cell death and possible liver failure (Holt and Ju, 2006).

3.3.1 Cellular Mediated DILI

Direct mitochondrial inhibition occurs through uncoupling or inhibition of the mitochondrial respiratory chain resulting in ATP depletion and accumulation of reactive oxygen species (ROS) which lead to mitochondrial permeability transition (MPT) resulting in the apoptosome formation. The apoptosome stimulate the cell death via chromatin

condensation and nuclear fragmentation (Russmann et al., 2009). Specific immune responses can be evoked through the binding of the drug or its metabolite to Human Leukocyte Antigen (HLA) proteins, which are then presented to T cells and recognized as antigens. The neo-antigens are subsequently placed on antigen presenting cells to activate formation of antibodies against themselves or activate the immune system to form auto-antibodies against cell structures (Robin et al., 1997).
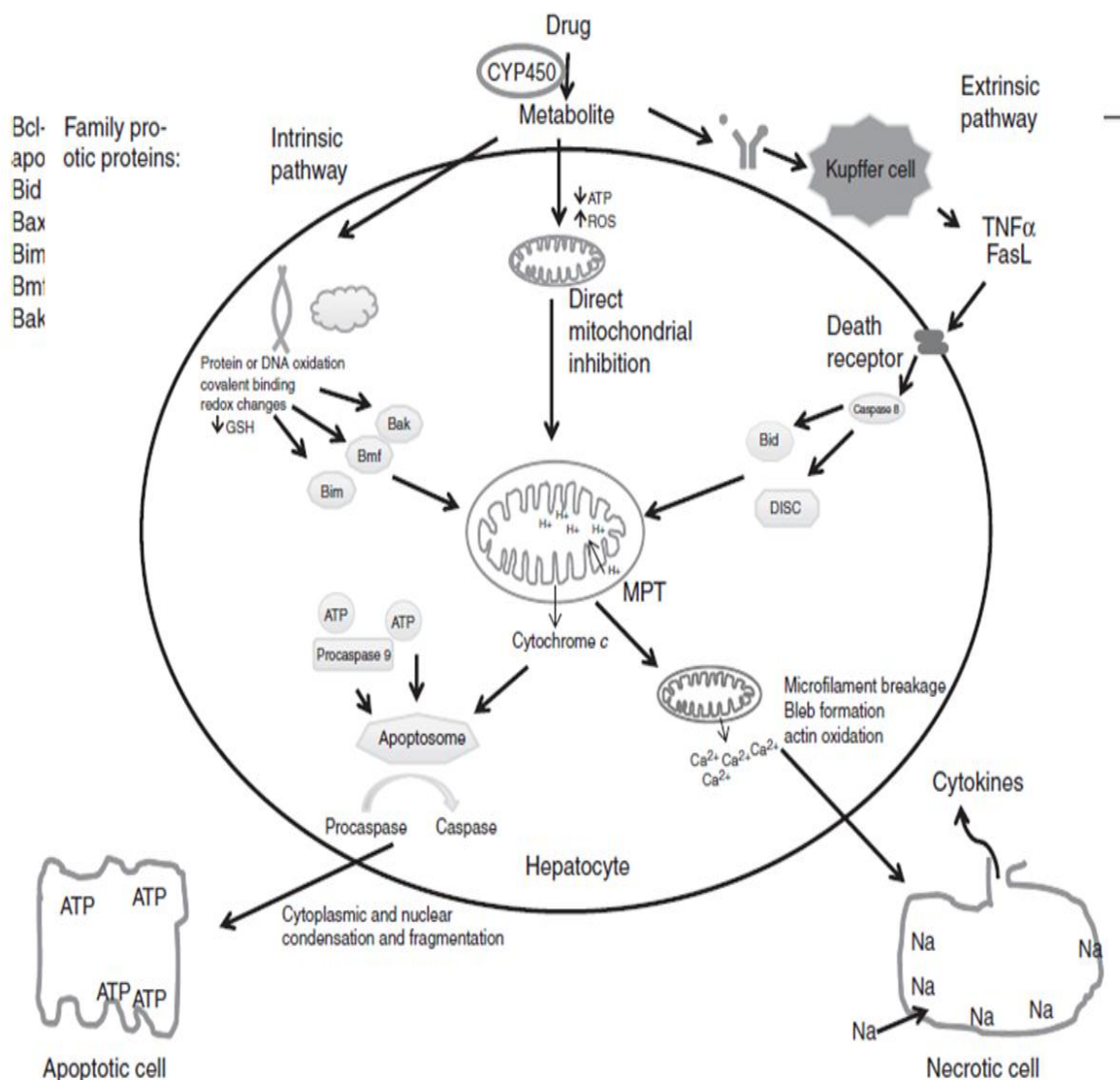
Figure 3.1 Mechanisms of Drug Induced Liver Injury (Au et al., 2011)

Toxic metabolites are generated from parent compounds via CYP450 and then affect mitochondrial damage via one of three pathways: intrinsic, extrinsic, or direct mitochondrial inhibition. Mitochondrial permeability transition (MPT) results in mitochondrial membrane disruption. In the presence of ATP, an apoptosome is formed and the cell is degraded via fragmentation and apoptosis. In the absence of ATP, led to increased mitochondrial

membrane permeability results in increased cytosolic calcium and sodium, cell lysis, necrosis and cytokine release (Au et al., 2011).

Cell stress initiates the direct pathway (Mitochondrial stress) in which proapoptotic proteins are activated and anti-apoptotic proteins are inhibited result into activation of mitochondrial permeability transition (MPT). Immune reactions activate the extrinsic pathway, where antigen presentation causes Kupffer cells to release Tumour Necrosis Factor - α (TNF-α) and FAS ligand (FasL). TNF-α and FasL then bind to intracellular death receptors and death domain proteins to activate caspase 8 resulting in the formation of the death inducing signaling complex (DISC). Caspase 8 also activates the Bcl-2 pro-apoptotic proteins which, in conjunction with the DISC complex, lead to MPT (Kaplowitz, 2002; Grattagliano et al., 2009).

The final step of injury involves either cellular apoptosis or necrosis. Apoptosis is an ATP-dependent pathway and can only occur if MPT does not occur rapidly and simultaneously in all cellular mitochondria. When ATP is present, the cytochrome C will bind a cytoplasmic scaffold protein and pro-caspase 9 to form an apoptosome, which serves to activate caspases resulting in cytoplasmic and nuclear condensation and fragmentation. MPT disrupts mitochondrial membranes by increasing permeability and proton influx through the inner membrane therefore disturbing ATP synthesis. This perturbation also causes mitochondrial matrix expansion and increased permeability of the outer mitochondrial membrane with release of cytochrome C and other pro-apoptotic proteins into the cell cytoplasm (Malhi et al., 2008). The fragments are then removed by phagocytosis. The process of apoptosis occurs without loss of plasma membrane integrity, which greatly decreases inflammation causing minimal secondary damage (Malhi et al., 2008).

Necrosis is the result of severely compromised mitochondrial function by MPT and depletion of ATP. The result is severe disruption of cell processes, which is followed by bleb formation, actin oxidation, microfilament breakage, cellular swelling and eventually plasma membrane rupture (Kaplowitz, 2002). The lysis process includes the release of cytokines, which can amplify the injury to surrounding hepatocytes (Russmann et al., 2009).

3.3.2 IMMUNE MEDIATED DILI

Specific immune responses can be evoked through the binding of the drug or its metabolite to HLA proteins, which are then presented to T cells and recognized as antigens. The neo-antigens are subsequently placed on antigen presenting cells to activate formation of antibodies against themselves or activate the immune system to form auto-antibodies against cell structures (Robin et al., 1997).

The metabolite of drug tends to release of the inflammatory mediator (TNF- α, IL-6, IL-2, IL-18, and IFN-ϒ) from Kupffer cell and natural killer cell. This inflammatory mediator led to the tissue damage. The formation of protein and drug adduct within the cell lead to cellular infiltration of lymphocyte which causes hepatitis and liver inflammation. Drugs suspected to induce these types of immune mediated adverse drug reactions include Halothane, Dihydralazine, Diclofenac, Phenytoin, And Carbamazepine (Uetrecht et al., 1999).

3.4 PATHOLOGICAL CONDITION ASSOCIATED WITH DILI

3.4.1 Hepatocellular Necrosis

Hepatocyte necrosis may be a sequela of direct toxicity or immune mediated damage. Cellular disintegration is caused by injury to plasma membranes and organelles. Apoptosis (programmed cell death) is also seen in DILI. Hepatocellular necrosis can range in severity from asymptomatic increases of aminotransferase enzymes to jaundice to overt hepatic failure (Zimmerman et al., 1978). With intrinsic hepatotoxins, nonspecific gastrointestinal symptoms such as nausea or vomiting typically begin within a few hours of exposure. Necrosis may predominantly involve a particular liver Zone because the enzymes involved in drug metabolism are often zonally distributed (e.g. cytochrome P450 2E1, involved in Paracetamol metabolism, is predominantly in (Zone 3 centrilobular) or because toxicity depends on the oxygen gradient across liver Zones. Non-zonal necrosis is usually immunologically mediated. The clinical manifestations of necrosis depend on its extent and duration. Recovery after sub-massive zonal necrosis usually occurs without significant scarring, but non-zonal necrosis can lead to nodular scarring (Zimmerman et al., 1999).

3.4.2 Steatosis

The exposure to drugs can result in accumulation of fat droplets within hepatocytes i.e. known as steatosis. Microvesicular Steatosis result of direct toxicity on the mitochondria and their oxidative processes. Example which causes Microvasicular Steatosis included Tetracycline, Sodium Valproate and Aspirin etc. Macro vesicular steatosis caused due to triglyceride accumulation, due to defects in lipoprotein metabolism, damage to plasma membrane or increased lipid delivery to hepatocytes consequent on increased synthesis or mobilization. Some drugs (e.g. Minocycline) produce both microvesicular and macrovesicular steatosis. Chronic steatosis with Mallory bodies (usually seen in alcoholic liver disease) is seen with Nifedipine, Corticosteroids and Tamoxifen (Zimmerman et al., 1999). Phospholipidosis (phospholipids accumulated in lysosomes as a result of inhibition of phospholipases) is seen with Thioridazine and Chlorphenamine. Some drugs (e.g. Amiodarone) can cause both chronic steatosis and phospholipidosis (Zimmerman et al., 1999).

3.4.3 Cholestasis

Cholestasis may occur because of selective interference in bile formation and flow (e.g. Oestrogens and Ciclosporin) immunogenic or direct toxic effect on biliary canaliculi of the drug; or accumulation of toxins consequent upon drug-induced inhibition of their secretion. The mechanism of drug-induced cholestasis has not been completely elucidated but is thought to involve interference with the function of one of the ATP-binding super family of canalicular transporters such as Bile salt export pump (BSEP), Multidrug resistance protein 3 (MDR3) and multi-drug resistance protein 2 (MRP2) (Pauli-Magnus et al., 2006). This can lead to benign cholestasis with little or no liver injury. Patients who develop cholestasis classically present with pruritus, pale stools and dark urine. Example which causes Cholestasis included Chlorpromazine, Fusidic Acid and Cyclosporin etc.

3.4.4 Vascular/sinusoidal

Drugs may affect the vascular endothelium, liver sinusoidal structures or clotting system. Hepatic vein thrombosis may occur in predisposed oral contraceptive users, often as a result of latent myeloproliferative disorders. In veno-occlusive disease and nodular regenerative hyperplasia, there is direct acute or chronic injury to the venular endothelium and Zone 3 hepatocytes. Veno-occlusive disease is a severe form of drug-induced liver injury characterized by thrombosis of efferent hepatic venules, leading to centrilobular necrosis and liver outflow obstruction, which can progress to congestive cirrhosis, as outlined in Table (Zimmerman et al., 1986; Zimmerman et al., 1993). The condition presents clinically with abrupt onset of severe abdominal pain, hepatomegaly, and jaundice, accompanied by extreme increases of hepatic aminotransferase and alkaline phosphatase enzymes (McDonald et al., 1993). Veno-occlusive disease can progress rapidly to overt hepatic failure, with manifestations of congestive cirrhosis such as ascites, coagulopathies, and hepatic coma. Injury to sinusoidal supporting structures leads to peliosis hepatitis, sinusoidal dilatation or hepatoportal sclerosis (a toxic effect of vitamin A) (Zimmerman et al., 1999).

3.4.5 Hepatic Tumors

Chronic use of oral contraceptives is associated with the development of hepatic adenomas, benign tumors typically observed only in women of childbearing age and which were exceedingly rare before the widespread use of these agents (Edmondson et al., 1976; Zimmerman et al., 1993). These tumors usually resolve completely with drug withdrawal, and risk of development is highly correlated with duration of drug exposure (Edmondson et al., 1977).

3.4.6 Toxic Hepatitis

Hepatocellular necrosis is a hallmark of these injuries, but the associated symptoms and histologic pattern of injury are nearly identical to those observed with acute viral hepatitis. (Scheuer et al., 1988; Bass et al., 1993; Zimmerman et al., 1993; Pande et al., 1996; Barnard et al., 1996). Histologically, these injuries typically reflect diffuse hepatocellular necrosis,

which may be associated with cholestasis. Lobular structure is generally maintained, and even in severe cases, areas of necrosis are usually surrounded by viable hepatocytes that reveal various degrees of degenerative change (Zimmerman et al., 1993). Extreme increases of hepatic aminotransferase enzymes, jaundice, and coagulopathies are associated withtoxic hepatitis some of the example includes- Isoniazid, Phenytoin, Sulfonamides.

3.5 Criteria for the Diagnosis of DILI

The main challenge is to establish a causal relationship between a certain medication and liver injury; several clinical scales have been developed. The scoring criteria are based on the chronological relationship between drug intake/drug withdrawal and clinical effect, clinical course of reaction, exclusion of other potential causes, and re-challenge. The Rousse Uclaf Causality Assessment Method of the Council of International Organization of Medical Science (RUCAM/CIOMS) is the most frequently used criteria set for the diagnosis of DILI (Tajiri K et al., 2008). In addition to the aforementioned criteria, the RUCAM/CIOMS scale scores several risk factors (age, alcohol consumption, and pregnancy) and separates DILI into the three patterns described above: hepatocellular, cholestatic, and mixed (Benichou, 1990; Danan et al., 1993).

The Maria and Victorino (M&V) Scoring system simplifies the approach by using only five of the seven criteria of the RUCAM/CIOMS scale, but also considers the presence of extra hepatic manifestations such as fever, rash, arthralgia, eosinophilia, or cytopenia. (Maria VA et al., 1997) A major critique of the M&V scale is the omission of the liver injury pattern. In addition, the M&V scale is not sensitive to diagnosing chronic forms of DILI and fulminant drug-induced hepatitisee (Aithal, 2000; Lucena et al., 2000).

In clinical practice these scales are not consistently used for the diagnosis of DILI. In order to obtain better data concerning drug hepatotoxicity and to provide access to a case registry, the National Institutes of Health (US) has sponsored an on-going research titled the Drug-Induced Liver Injury Network (DILIN) (Hoofnagle et al., 2004; Bell and Chalasani, 2008; Fontana et al., 2009).which is monitoring and controlling the incidence of the ADR's in world wide. In April 2010, the US Food and Drug Administration (FDA) had added a boxed

warning, the strongest warning issued by the FDA, to the prescribing information for Propylthiouracil. The boxed warning emphasizes the risk for severe liver injury and acute liver failure, some of which have been fatal. The boxed warning also states that Propylthiouracil should be reserved for use in those who cannot tolerate other treatments such as Methimazole, Radioactive Iodine, or Surgery (Mehta, 2013).

3.6 Risk Factor Associated with DILI

Idiosyncratic drug-induced liver injury (DILI) is a rare disorder that is not related directly to dosage and little is known about individuals who are at increased risk. There are no suitable preclinical models for the study of idiosyncratic DILI and its pathogenesis is poorly understood. The prevalence of DILI is more likely toward the type I adverse drug reaction rather than idiosyncratic reaction but now so many drug causes this reaction lead to poor diagnostic as well as treatment follow-up. It is likely to arise from complex interactions among genetic, nongenetic host susceptibility, and environmental factors. Nongenetic risk factors include age, sex, and other diseases (e.g., chronic liver disease or human immunodeficiency virus infection). Compound-specific risk factors include daily dose, metabolism characteristics, and drug interactions. Alcohol consumption has been proposed as a risk factor for DILI from medications, but there is sufficient evidence. Many studies have explored genetic defects that might be involved in pathogenesis and focused on genes involved in drug metabolism and the immune response (Chalasani et al., 2010).
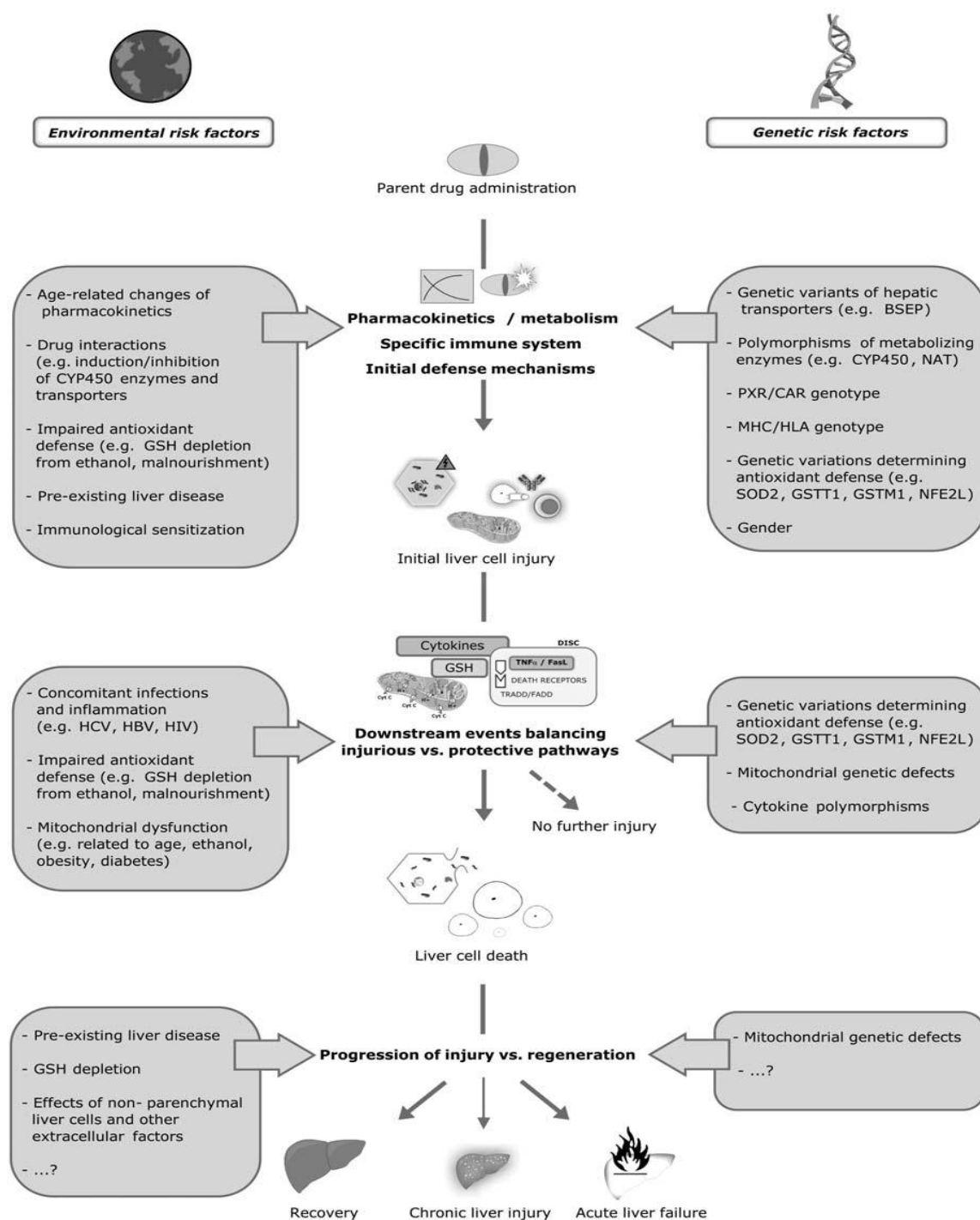
Figure 3.2 Risk Factor Associated With DILI (Russmann et al., 2009)

Risk factors can be classified into environmental vs. genetic factors. From a mechanistic point of view risk factors can further affect all different levels of events leading to the final

outcome of drug-induced liver injury, which is mostly dichotomous, i.e. full recovery vs. acute liver failure.

3.6.1 NONGENETIC RISK FACTORS

3.6.1.1 Age

Age is a risk factor for DILI, but only from specific medications. (Larrey, 2002) Younger age is a risk factor for certain medications such as Valproic Acid and for Reye syndrome, associated with aspirin use. As age increases, so does the risk of liver injury from compounds such as Erythromycin, Halothane, Isoniazid, Nitrofurantoin, and Flucloxacillin has been increases (Zimmerman, 1999). The risk of hepatotoxicity from isoniazid increases significantly with age (Jinjuvadia et al., 2007). In a large study of patients in a US tuberculosis clinic, the age-specific incidence of isoniazid hepatotoxicity was 4.4 per 1000 patients age 25–34 years, whereas it increased to 20.83 per 1000 patients age 50 years and older (Fountain et al., 2005). Increasing age also increases the risk for hepatotoxicity from amoxicillin/ clavulanate (Lucena et al., 2006). The cholestatic type of DILI is more common among the elderly, whereas hepatocellular DILI appears to be more common in younger individuals (Andrade et al., 2005; Lucena et al., 2009).

The reasons that age affects DILI phenotypes are unclear. Although older age can affect the clearance of certain CYP3A substrates, (Cotreau et al., 2005) older age does not significantly alter the activity or expression of phase I or phase II drug metabolizing enzymes. Renal function is impaired in the elderly, who might increase drug concentrations in the liver; liver volume and liver blood flow have been correlated inversely with age. However, in the elderly these physiologic alterations would account for intrinsic DILI rather than idiosyncratic DILI.

It is unclear if the elderly produce more reactive metabolites or have increased immune response to these metabolites. The increased risk of hepatotoxicity from some drugs might result from polypharmacy among the elderly. Although the incidence of certain adverse effects can increase with the use of multiple medications, there is little evidence to support

polypharmacy as a predisposing factor for DILI. Combinations of 2 or more hepatotoxic drugs increased the risk for DILI by a factor of 6 in one study. However, a subsequent prospective study did not show a significant relationship between polymorbidity or polypharmacy and the risk for DILI (Chalasani et al., 2010).

3.6.1.2 Sex

Women are believed to be at higher risk for idiosyncratic DILI than men, based on a higher prevalence of women in published DILI studies. In a landmark prospective study, (Lucena et al., 2009) reported that of 603 patients with DILI, 51% were male and 49% were female. Similarly, a population-based study reported an annual incidence of 10.4-3.0 per 100,000 women and 17.1-3.6 per 100,000 men. In this study, the standardized female to male incidence ratio increased from 0.86 (0.26 –2.90) in women younger than 50 years of age to 2.62 (1.0 – 6.92) in women older than age 50. Women are more susceptible to liver injury associated with Halothane, Flucloxacillin, Isoniazid, Nitrofurantoin, Chlorpromazine, or Erythromycin whereas men have an increased risk of azathioprine-induced liver injury. (Zimmerman et al., 2000) observed that autoimmune-type DILI occurred almost exclusively in women. A prospective trial of the DILI network reported a significantly greater number of women with hepatocellular DILI than men (65% vs 35%; $P \geq 0.05$) (Bjornsson et al., 2008).

3.6.1.3 Daily Dose

There is a traditional view that idiosyncratic DILI cannot be predicted based on dose, although a relationship to dose was observed for some medications, such as Diclofenac, Amoxicillin/Clavulanate, and Flucloxacillin. Idiosyncratic liver injury associated with Bosentan also has been shown to have dose dependency (Kenyon et al., 2003). There are case reports in which dose reduction led to improvement and less occurrence of hepatotoxicity caused by Mianserin (Otani et al., 1998).

3.6.1.4 Metabolism Characteristics

In associating the risk of DILI with hepatic metabolism of 207 of the most commonly prescribed oral medications in the United States, compounds with 50% or greater hepatic metabolism caused a significantly higher frequency (compared with drugs with less hepatic metabolism) of alanine aminotransferase (ALT) levels greater than 3 times the upper limit of normal (34% vs 10%; P>0.007), liver failure (28% vs 9%; P> .001), liver transplantation (9% vs 1%; *P* -.045), and fatal DILI (23% vs 4%; P> .0003), but not jaundice (43% vs 34%; P>0.2).Twelve compounds with no hepatic metabolism (Risedronate, Alendronate, Hydrochlorothiazide, Nadolol, Cefdinir, Cefprozil, Gabapentin, Metformin, Cephalexin, Benzonatate, Cefuroxime, and Sotalol) were not found to causeliver failure, liver transplantation, or fatal DILI (Lammert et al., 2010).

3.6.1.5 Drug Interactions

The formation of toxic reactive metabolites during hepatic metabolism is believed to be the main pathogenic mechanism for DILI. Most drugs are bio-transformed by phase I and/or phase II metabolic reactions. Oxidation, Reduction, and Hydrolytic Reactions are included in the phase I reactions, whereas phase II reactions include Conjugation reactions and involve either esterification of the parent compound or a metabolite created by the phase I reactions. There are more than 20 different CYPs in the CYP450 family in human liver. CYPs are responsible for the most phase I reactions, and formation of reactive intermediates is more abundant in the centrilobular Zone than in the periportal Zone (Chalasani et al., 2010).

Centrilobular necrosis is one of the characteristic features of severe DILI, so drug-metabolizing enzymes might mediate the pathogenesis of DILI. Certain drugs can modify the hepatotoxic potential of other drugs by enzyme induction and lead to formation of reactive metabolites. Examples of Cytochrome P (CYP) enzyme inducers are Rifampicin, Phenytoin, Isoniazid, Carbamazepine, Smoking, and Ethanol. Some drug metabolizing and detoxification pathways might increase the risk for hepatotoxicity from other drugs, but there is little in vivo evidence to support this model. In a meta-analysis of studies of hepatotoxicity from Isoniazid and Rifampicin, the incidence of liver injury was significantly greater among patients who

received this drug combination than those who received either as a single agent. Rifampicin, which can induce microsomal enzymes, seems to increase the risk of liver injury from Isoniazid in frequency and latency. Pyrazinamide was reported to increase the hepatotoxicity of Isoniazid (Steele et al., 1991).

3.6.1.6 Alcohol Consumption

The effects of acute and chronic alcohol use on the risk of Acetaminophen hepatotoxicity are well established, the role of Alcohol in idiosyncratic DILI is less clear. Alcohol consumption is one of the criteria in the Rousse Uclaf Causality Assessment Method (RUCAM) causality assessment instrument; although there is no evidence that alcohol consumption increases the risk of liver injury from medications other than Methotrexate, Isoniazid, or Halothane (Chalasani et al., 2010). Consumption of large amounts of alcohol increases the risk of fibrosis/ cirrhosis in long-term users of Methotrexate. However, Methotrexate alone might not cause severe liver fibrosis, other risk factors such as Diabetes Mellitus Type 2, overweight, and heavy use of Alcohol might contribute. Chronic Alcohol abuse might increase the hepatotoxicity of Antituberculosis (Anti-TB) drugs possibly from Alcohol-mediated induction of hepatic CYP2E1 (Whiting-O'Keefe et al., 1996).

3.6.1.7 Underlying Disease States

There is controversy about whether chronic liver disease increases the risk for DILI. There is a belief that patients with chronic liver disease and cirrhosis are not necessarily prone to DILI but patients with preexisting liver disease are at higher risk for complicated courses and adverse outcomes from DILI. A limited number of studies systematically have evaluated the safety of drugs in patients with pre-existing liver disease. Patients with increased baseline levels of aminotransferases have an increased risk of statin-induced hepatotoxicity. Several other studies confirmed the safety of statins in patients with chronic liver disease, including nonalcoholic fatty liver disease and hepatitis C (Khorashadi et al., 2006).

3.6.2 GENETIC RISK FACTORS

Because of their rarity and unpredictability, idiosyncratic DILI events are considered to have a strong genetic basis but significant association between certain genetic traits and DILI has been shown for only a few compounds. Even when such an association has been observed, generally the odds ratio (OR) of a given haplotype to increase the risk of DILI has been rather low (With the exception of flucloxacillin) (Russmann et al., 2010).

DILI is likely a complex genetic disorder in which multiple genetic variants along with environmental risk factors are responsible for liver injury. Several organizations (DILI network, DILIGEN, Spanish Hepatotoxicity Registry, and Serious Adverse Event Consortium) are collecting genomic DNA from patients with well-phenotype DILI and novel observations are starting to emerge from their work (Donaldson et al., 2008; Daly et al., 2009).

3.6.2.1 Variations in Phase 1 Drug-Metabolizing Enzymes

Formation of toxic reactive metabolites by cytochrome P450 enzymes (CYPs) is considered to be required for the pathogenesis of DILI. There is considerable interindividual variation in the activity of different CYPs and many CYPs are polymorphic.CYP3A is the most abundant and most important phase 1 enzyme; it consists of 3 isoforms: 3A4, 3A5, and 3A7. CYP3A4 is not polymorphic whereas CYP3A5 and CYP3A7 are polymorphic in nature. There is no evidence to associate variations in CYP3A with DILI. CYP2C9 is an important phase 1 enzyme involved in the metabolism of several important therapeutic agents such as nonsteroidal anti-inflammatory drugs, phenytoin, and warfarin. CYP2C9 shows functional polymorphism (CYP2C9*1,*2, and *3); some polymorphisms initially were associated with diclofenac hepatotoxicity, but subsequent studies failed to confirm any significant association. CYP2C19 is another important phase 1 enzyme that also has functional polymorphisms. It metabolizes proton pump inhibitors, antidepressants, and antiepileptics (Chalasani et al., 2010).

3.6.2.2 Variations in Phase 2 and Detoxifying Enzymes

N-acetylation is an important phase II reaction and N-acetyltransferase 2 (NAT2) has been implicated in DILI. NAT2 is highly polymorphic and there is interindividual variability in its metabolic activity. NAT2*4 has the highest acetylation activity, whereas *5,*6, and *7 reduced enzymatic activity. Slow rates of acetylation have been associated with increased risk of hepatotoxicity from sulfonamides and isoniazid (Mitchell et al., 1975). Isoniazid is metabolized primarily in the liver, initially by NAT2 into acetyl-isoniazid; this is hydrolyzed rapidly into acetyl-hydrazine, which is either oxidized by CYP2E1 into toxic reactive metabolites or acetylated to form diacetyl-hydrazine. "Slow acetylators" presumably do not detoxify acetyl-hydrazine rapidly, promoting oxidation by CYP2E1 into toxic intermediaries (Russmann et al., 2009).

3.6.2.3 Hepatobiliary Transporters

Hepatic detoxification of xenobiotics results in their anionic conjugates with glutathione, sulfate, and glucuronate. These conjugated xenobiotics become substrates for hepatic drug transport, another potential step for drug hepatotoxicity. Drug metabolites actively are transported across hepatocyte membranes by transporters (uptake or efflux transporters) on the canalicular or the apical membranes. In the liver, transport at the basolateral membrane involves the organic anion transporting polypeptide and in the organic anion transporter. Limited data exist on defects in these uptake transporters and associated hepatotoxicity. The inhibition of efflux proteins can lead to cholestatic liver injury caused by certain compounds or their metabolites. The efflux of drugs into bile involves canalicular transporters of the Multidrug Resistance-Related Protein (MRP) family, which includes the glycoproteins: ATP-binding cassette-1 Protein (ABCB1), ATP-binding cassette-4 gene (ABCB4), Multidrug Resistance-Related Protein-2 (MRP2) ATP-binding cassette, sub-family C gene (ABCC2), and bile salt export pump (BSEP), ATP-binding cassette-11 gene (ABCB11). Drugs that inhibit export on the canalicular side through inhibition of BSEP can lead to cholestasis in susceptible subjects. Cholestatic liver injury from Sulindac, Flucloxacillin, Terbinafine, and

Bosentan has been associated with inhibition of the canalicular BSEP (Pauli-Magnus et al., 2006).

3.6.2.4 Association between DILI and Specific Human Leukocyte Antigen (HLA) Haplotypes

There is evidence for an association between polymorphisms in HLA class II antigens and adverse drug reactions; specific HLA haplotypes have been associated with nonhepatic and hepatic adverse reactions. An association between HLA-B*5701and Abacavir hypersensitivity led to the concept that prospective testing for this allele might minimize the risk of Abacavir hypersensitivity (Mallal et al., 2008).

3.6.2.5 Dysregulation of Cytokines

Dysregulation of cytokine production also might mediate the pathogenesis of DILI in a non–medication-specific manner. The polymorphisms in interleukin (IL)-10, IL-4, and IL-4 receptors in 24 patients with Diclofenac-induced liver injury, Diclofenac-exposed controls, and as many as 321 healthy controls; they observed a higher frequency of IL-10 and IL-4 variants in patients with Diclofenac- induced DILI compared with controls. The investigators speculated that polymorphisms that increase IL-10 or reduce IL-4 expression might contribute to a T-helper cell 2–mediated antibody response to diclofenac-induced neoantigens (Pachkoria et al., 2006).

3.6.2.6 Mitochondrial DNA Mutations

Some drugs (e.g. Valproate, Salicylate, and Antiretroviral Agents) cause liver injury through mitochondrial toxicity; a recent preliminary study associated mitochondrial DNA mutations with liver injury caused by some compounds. Seventeen patients with suspected Valproate hepatotoxicity enrolled in the DILI network studies were assessed for genetic variations in the mitochondrial DNA polymerase gamma gene (POLG) (Day et al., 2009).

3.7 DRUG THAT CAUSES LIVER INJURY

3.7.1 Analgesics

The development of liver failure from acetaminophen is dose dependent; hepatic failure is more likely with ingested dosages >150 mg/kg. Excessive acetaminophen overwhelms the normal conjugation pathways of sulfation or glucuronidation, mandating that acetaminophen is metabolized by the alternate pathways through cytochrome P450. N-Acetylp benzoquinoneimine is produced, which is highly reactive and depletes glutathione. Accumulation of N acetyl- p-benzoqinoneimine leads to cell death and hence hepatocellular necrosis. Histologically, there is pericentrivenular cellular necrosis. Clinical features of acetaminophen-induced hepatotoxicity include anorexia, nausea, and vomiting. In severe ingestion, hypoglycemia and lactic acidosis are prominent early features (Jack et al., 2010).

3.7.2 Anesthetic

Halothane-induced liver injury is an example of immune-mediated hepatotoxicity, and both hepatitis and acute liver failure have been reported in children (Pratilas et al., 1978; Kenna et al., 1987). The underlying mechanism for liver damage is the formation of triflouoroacetyated proteins by cytochrome P450. Approximately 20% of halothane is converted by cytochrome 450, predominantly cytochrome 2E1, to the unstable intermediate trifluoroacetyl chloride. This intermediate binds to liver proteins, causing cellular injury (Pratilas et al., 1978), but in some patients there is also an immune response to cytochrome 2E1. In both animal and human studies, the production of antibodies against specific human cytochrome P450 2E1 has been demonstrated and forms a diagnostic test for halothane hepatitis (Pratilas et al., 1978).

3.7.3Antitubercular Drug

The exact pathogenesis of the Isoniazid induced hepatotoxicity is not well understood. The histopathology resembles that of viral hepatitis, although a toxic metabolite of the drug, monoacetyl hydrazine, is likely responsible for hepatocyte death. Monoacetyl hydrazine is

largely excreted by the kidneys after further conversion, but it may also be converted to electrophilic intermediates by the P450 system; these intermediates are hepatotoxic (Steele et al., 1991).

3.7.4 B-Lactam Antibiotics

Ampicillin and amoxicillin cause liver injury at an incidence of 0.3/10,000 prescriptions. However, when amoxicillin is combined with clavulanic acid (ACA), the incidence of injury increases to 1.7/10,000 prescriptions (Eliasson et al., 1996). The ACA-associated hepatotoxicity is most commonly a delayed Cholestatic or mixed hepatocellular Cholestatic liver injury pattern. Age may be the most important determinant in the biochemical expression of ACA hepatotoxicity; cholestatic/mixed injury is more common in older adults, whereas younger age is associated with cytolytic damage (Lucena et al., 2006).

3.7.5 Tetracycline Antibiotics

The Tetracycline's have been implicated in dose-related hepatotoxicity, especially in women, during pregnancy, and in individuals with renal disease (Garcia Rodriguez et al., 1996). The overall incidence of hepatotoxicity due to Minocycline is 1.04/ 10,000 exposed person–months, and for that due to Oxtetracycline/ Tetracycline is 0.69/10,000 exposed person–months. With high doses of the Tetracycline's, clinical evidence of hepatotoxicity appears 4 to 6 days into therapy and is characterized by nausea, vomiting, abdominal pain, and mild jaundice, with serum aminotransferase levels as high as 10 times the upper limit of normal and significant elevations of the serum amylase level. Chronic cholestasis is less common, but vanishing bile duct syndrome has been associated with tetracycline and doxycycline (Hunt et al., 1994).

3.7.6 Macrolide Antibiotics

The incidence of Erythromycin-induced liver disease is not definitely known, but in adults it seems to be between 2.28 cases/million patients treated for 10 days and 3.6/ 100,000 patients, with similar risks from any of the erythromycin salts (Derby et al., 1993; Garcia

Rodriguez et al., 1996). An immunoallergic mechanism of drug toxicity is favored by the clinical features, which include peripheral eosinophilia, a delay in the initial onset of hepatotoxicity yet a prompt onset within days upon re-challenge, and symptoms of rash and fever (Diehl et al., 1984; Pessayre et al., 1985).

3.7.7 Antiepileptic Medications

Abnormal liver enzymes have been reported in association with Chloral Hydrate, Clonazepam, Diazepam, Mephenytoin, Primidone, and Sultiam. However, these drugs are not considered to induce serious liver disease. The few reports of hepatic damage from phenobarbital include hepatocellular and cholestatic liver injury and also hypersensitivity reaction (Li et al., 1992). Hepatocellular damage from phenytoin is rare but an important possible side effect. In most cases, elevation of liver enzymes is benign and is caused by hepatic enzyme induction, but acute and Cholestatic hepatitis may occur. Valproic acid well tolerated, but benign elevation of any liver enzyme may occur in as many as 20% of patients.

3.7.8 Recreational Drugs

Marijuana (Cannabis sativa) and hashish (Cannabis indica) have more than 400 chemical compounds and commonly cause abnormalities of aspartate aminotransferase, alanine aminotransferase, or alkaline phosphatase, but serious hepatotoxicity and ALF have not been reported. Cocaine can cause ischemic necrosis of the liver, but there are no systematic reports of other hepatotoxic reactions, as far as we are aware (Murray et al., 2008).

3.7.9 Herbal Medications

Herbal medicines can have significant drug interactions with conventional drugs after induction of P450 enzymes (Favreau et al., 2007). The herbal medicines reported with hepatotoxic reactions are: Amanita Phalloides, Pyrrolizidine Alkaloids (Crotolaria, Heliotropium), and Chinese Herbal Medicines Jin Bu Huan (LycopodiumSerratum), Ma-Huang (Ephedra Alkaloid), PaeoniaSpp, DictamnusSpp, Lingzhi (GanodermaLucidum), Shou-Wu-Pian (PolygonumMultiflorum), Germander etc.

3.7.10 Slimming Agents

Weight loss agent's Ma Huang (Ephedra Alkaloid), Usnic Acid (Lichen Alkaloid), Chaso, and Onshido can lead to acute liver failure (Neff et al., 2004). One series has described 6 patients in whom acute hepatitis developed and 1 in whom ALF developed while they were using LipoKinetix (a mixture of Norephedrine, Sodium Usniate, Di-Iodothyronine, Yohimbine, And Caffeine) (Favreau et al., 2002). In a recent series, 25% of patients (3 of 12) with ALF required liver transplantation (Neff et al., 2004).

3.8 LIVER REGENERATION (LR)

The fascinating aspect of the liver is the capacity to regenerate after injury or resection. A variety of genes, cytokines, growth factors, and cells are involved in liver regeneration. The exact mechanism of regeneration and the interaction between cells and cytokines are not fully understood. There seems to exist a sequence of stages that result in liver regeneration, while at the same time inhibitors control the size of the regenerated liver (Takahashi et al., 1991).

The disease itself may be associated by elevations of transcription factors and cytokines that induce proliferation of hepatocytes and non-parenchymal cells. These pathophysiologic responses of the liver may aggravate the pathologic process resulting in augmented fibrogenesis in cirrhosis and the accumulation of mutations in progenitor cells and proliferating hepatocytes, finally leading to the development of hepatocellular carcinoma (HCC) (Taub et al., 2003).

Adult liver only one in 1000 cells is in mitosis at any given time (Diehl et al., 1996). There are three main phases of liver regeneration. Cells are normally in a resting G_0 state. After partial hepatectomy (PH), all hepatic cells simultaneously undergo transition into G_1 phase almost immediately. Hepatocytes enter S phase (DNA synthesis) roughly 12 ± 15 h after partial hepatectomy. The G_2 phase and mitosis (M phase) follow 6 ± 8 h after PH. Nonhepatocyte replication is usually delayed by approximately 24 h as a result of prolongation of their G_1 phase. During this initial phase of regeneration most hepatocytes will replicate at least once. When the original mass $\pm$ volume ratio of the liver has been achieved, the hepatocytes revert to their quiescent G_0 phase. The rat liver cans almost double its size

within 48 h of partial hepatectomy. Once the original liver mass is restored, liver regeneration stops abruptly (Labrecque, 1994). In vivo, the periportal hepatocytes are the first to begin replication, initially without any corresponding sinusoids or matrix. Cell clusters develop and replication spreads to the pericentral regions. Hepatocyte replication decreases by day 4 after partial hepatectomy, and thereafter non-parenchymal cells begin to recreate normal lobular hepatic architecture by dividing the cell clusters into cell plates surrounded by vascular spaces, sinusoids and spaces of Disse.

3.9 Carbamazepine induced liver injury

Carbamazepine (CBZ) is a widely used anti-epileptic agent. CBZ develop severe, potentially life-threatening idiosyncratic reactions such as mixed Cholestasis, agranulocytosis, hepatitis and Stevens-Johnson syndrome (Pallock et al., 1987; Kaufman and Shapiro et al., 2000; Björnsson and Olsson et al., 2005).
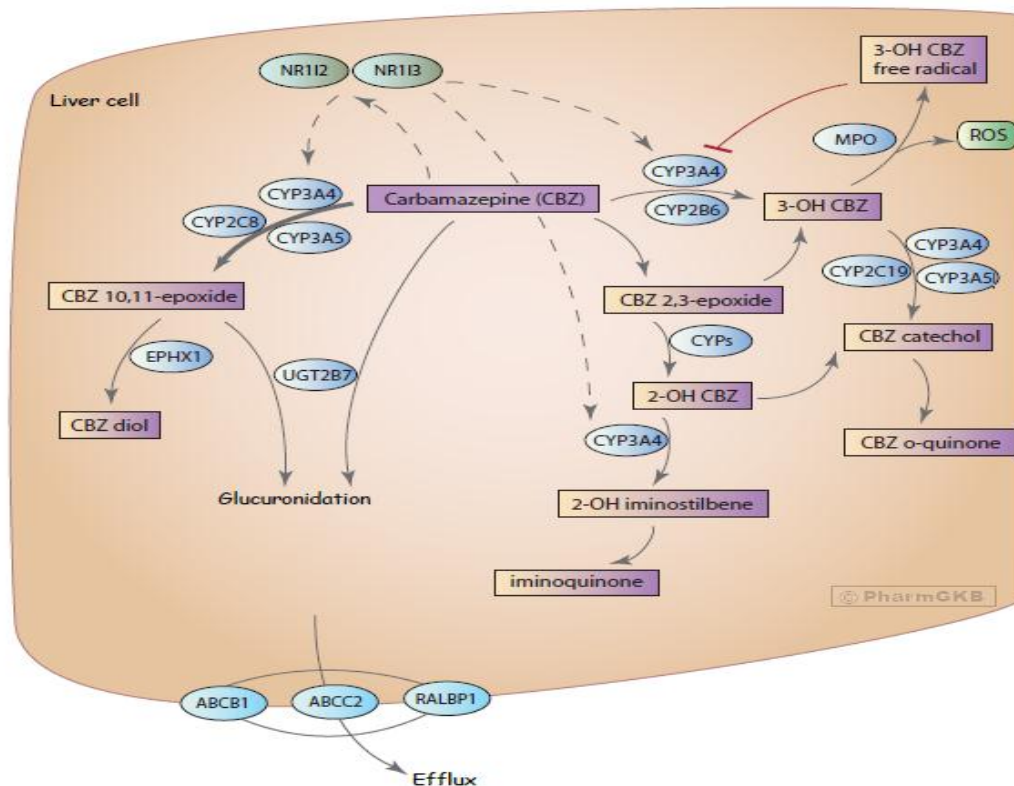
Figure 3.4 CBZ Metabolic Pathway (Thorn et al., 2011)

CBZ is metabolized by CYP3A4 into CBZ 10.11-epoxide (CBZ-E) than this is converted into the CBZ diol. The ring-hydroxylation of CBZ 10.11-epoxideled to formation of CBZ 2,3- epoxide, 2-hydroxy-CBZ (2-OH-CBZ) 3-hydroxy CBZ (3-OH CBZ), 2– OH iminostilbene and finally converted into the iminoquinone and 3- OH CBZ radical.

CBZ is almost completely metabolized in the liver with only around 5% of the drug excreted unchanged. The major route of metabolism is conversion to CBZ 10.11-epoxide (CBZ-E). This reaction is primarily catalyzed by CYP3A4 although CYP2C8 also plays a role, and involvement of CYP3A5 has also been suggested. Minor metabolic pathways include ring-hydroxylation to form 2-hydroxy-CBZ (2-OH-CBZ) and 3-hydroxy CBZ (3-OH CBZ). The formation of each presumably proceeds via an epoxide intermediate (referred to as an arene oxide intermediate), with CYP2B6 and CYP3A4, being the major catalysts of 3-OH-CBZ formation, and multiple CYPs involved in 2-OH-CBZ formation. Secondary metabolism of 2-OH-CBZ and 3-OH-CBZ by CYP3A4 represent two distinct potential bioactivation pathways. CYP3A4-dependent secondary oxidation of 2-OH-carbamazepine leads to the formation of thiol-reactive metabolites via an iminoquinone intermediate, whereas CYP3A4-dependent secondary oxidation of 3-OH-CBZ results in the formation of reactive metabolites capable of inactivating CYP3A4 and forming covalent adducts. 3-OH CBZ, and to a lesser extent 2-OH CBZ and CBZ, can be metabolized to form radicals by myeloperoxidase (MPO). This releases reactive oxygen species and may lead to the formation of protein adduct. Covalent binding and protein adduct formation has also been observed for another anti-epileptic drug, phenytoin, and is generally considered to be a necessary step in the pathogenesis of idiosyncratic reactions to this class of compounds (Thorn et al., 2011).

Serious CBZ-associated hepatotoxicity assumes the following two forms:

1) A hypersensitive reaction in the form of granulomatous hepatitis that presents with fever and abnormal liver functions.

2) An acute hepatitis and hepatocellular necrosis with fever and inflammation (Björnsson and Olsson et al., 2005; Björnsson et al., 2008).

However, there are no reports of CBZ-induced hepatotoxicity in an in vivo animal model. The presence of a specific autoantibody directed against a human liver microsomal protein in a patient who had severe hepatotoxicity with CBZ has been reported (Pirmohamed et al; 1992). Based on these reports, the liver injury associated with CBZ is thought to have an immunoallergic basis. Numerous studies have assayed CBZ-induced cytotoxicity by reactive

metabolites in vitro, and they have suggested that CBZ 2, 3-epoxide and 3-hydroxy (OH) CBZ might play a role in CBZ-induced idiosyncratic drug reactions via covalent binding to the protein or the production of reactive oxygen species (ROS) and glutathione (GSH) and microsomal epoxide hydrolase are involved in detoxification (Pirmohamed et al; 1992; Lu and Uetrecht; 2008).

It is well known that ROS is involved in drug-induced liver injury via mitochondrial dysfunction or hepatocyte necrosis (Jaeschke et al., 2002), and it was recently reported that ROS increased the expression of the toll-like receptor (TLR) and the receptor for advanced glycation end-products (RAGE), as well as their ligands, such as S100 protein and high-mobility group box 1 (HMGB1) (Yao and Brownlee et al., 2010). The activation of TLR or RAGE results in the induction of inflammatory cytokines and chemokines (Lotze et al., 2007). Cytokines and chemokines, followed by inflammation or the infiltration of lymphocytes to hepatocytes, are involved in immune-mediated hepatotoxicity, and they are predominantly secreted from immune cells such as T-lymphocytes and macrophages (Kita et al., 2001; Oo and Adams, 2009). Cytokines are generated by several transcriptional factors: T-box expressed in T cells (T-bet) induces the secretion of interferon (IFN)-γ and interleukin (IL)-12; GATA-binding domain-3 (GATA-3) induces IL-4, IL-5 and IL-13 production; and retinoid-related orphan receptor (ROR)-γt promotes the production of IL-6 and IL-23, which leads to increase in IL-17 generation (Kidd,2003; ; Langrish et al., 2005; Steinmaan et al., 2007). The IL-17 is involved in the pathogenesis of immune-mediated hepatotoxicities in mice, such as halothane- or α-naphthylisothiocyanate-induced hepatotoxicity (Kobayashi et al., 2009; Kobayashi et al., 2010).

4. MATERIALS AND METHODS

4.1 PROTOCOL APPROVAL

The protocol of the experiment has been approved by animal ethical committee as per the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India (FLAIR/January/2013/12, 27 January 2013). This work was carried out at Flair Labs, Opp-Gujarat Eco- Textile Park, NH -8, AT/PO: Palsana, Surat, - 394315 Gujarat, India.

4.2 ANIMALS

Female Swiss Albino mice of 8-10 weeks of age were chosen for the study and maintained under well-controlled conditions of temperature (22 ± 2 ºC), humidity ($55 \pm 5\%$) and 12h/12h light-dark cycle. Standard laboratory mice diet and UV-filtered water were provided *ad libitum*.

4.3 DRUG AND BICHEMICAL KITS

Carbamazepine was procured from Swati Chemicals, Phase-IV, Vatva, Ahmedabad - 382 445, Gujarat, INDIA. Biochemical kits were procured from Lab care diagnostics, Pvt. Ltd. India.

4.4 CARBAMAZEPINE INDUCED LIVER INJURY

Swiss albino mice were divided into 2 groups, and then further divide into 7 different groups for different time intervals (figure 4.1).

Table 4.1: Categorization of experimental animals in different groups

Groups	No. of Animals for different time intervals (hrs)						
	0	6	24	36	48	72	96
Vehicle Control (VC) (Corn oil, p.o.)	5	5	5	5	5	5	5
CBZ treated (CT) (350mg/kg, p.o-4 days & 800mg/kg, p.o-5th day)	6	6	6	6	6	6	6

4.5 INDUCTION OF CARBAMAZEPINE INDUCED DILI

Animals were maintained with free access to conventional dietary feed and water *ad libitum* throughout experimental period. All animals were monitored regularly for changes in body weight and mortality throughout the course of study. Carbamazepine was suspended in corn oil and given orally in the dose of 350mg/kg for four days and 800mg/kg on the fifth day in the CBZ treated animals. Whereas normal control animals were given only the vehicle i.e. corn oil. After 5th day of dosing, both blood samples were collected at different time intervals like- 0, 12, 24, 36, 48, 72, and 96 hrs. After collection of blood samples, the animals were sacrificed and liver was isolated. Blood samples were used for biochemical estimations and liver samples for biochemical analysis as well as histopathology.

4.6 SAMPLE COLLECTION AND ANALYSIS

Blood was collected from the retro orbital plexus of the animal while they were under light ether anesthesia. The blood was collected in sterile eppendorf tubes and allowed to clot for 10-15 minutes. Thereafter it was centrifuged at 4000 rpm for 10 minutes at 4°C. Serum was separated with the help of micropipettes into different eppendorf for further estimation of biochemical parameters i.e. Serum SGPT, SGOT and Glucose. For collection of liver tissue animals were sacrificed under ether anesthesia and abdominal cavity was opened. Whole liver was excised; extraneous tissues were separated, washed in saline and dried using filter paper. Left lobe was kept in 10% formaldehyde for histopathological analysis and other lobes were stored at -20°C until they were analyzed for tissue Glycogen content.

4.7 ESTIMATION OF VARIOUS PARAMETERS

4.7.1 SERUM BIOCHEMICAL ESTIMATION

4.7.1.1 SGPT (Serum Glutamic Pyruvic Transaminase):

The SGPT (or ALT) is a cellular enzyme, found in highest concentration in liver. High levels are observed in hepatic disease like hepatitis, diseases of muscles and traumatisms; its better application is in the diagnosis of the diseases of the liver.

PRINCIPLE:

Serum Glutamic pyruvic Transaminase catalyzes the reaction between alpha - ketoglutaric acid and alanine giving L-glutamic acid and pyruvic acid. Pyruvic acid, in the presence of lactate dehydrogenase (LDH) reacts with NADH giving lactic acid and NAD. The rate of NADH consumption is determined photometrically and is directly proportional to the SGPT activity in the sample.

PROCEDURE:

A working reagent was prepared by mixing Reagent I: (Buffer reagent) and Reagent-II: (Enzyme reagent) in the ratio of 4:1 respectively. The working reagent and the sample were mixed as mentioned below.

SAMPLE	100μL
REAGENT	1000μL

They were mixed well and allowed to stand for 1 minute at 37°C. Absorbance decrease per minute was measured during 3 minutes and the Δ A/min was determined.

CALCULATION:

Δ A/min. * 1746 = U/L of SGPT

4.7.1.2 SGOT (Serum Glutamic Oxaloacetic Transaminase):

The SGOT (or AST) is a cellular enzyme, is found in highest concentration in heart muscle, the cells of the liver. Although an elevated level of SGOT in the serum is not specific of the hepatic disease, is used mainly to diagnostic and to verify the course of this disease with other enzymes like SGPT.

PRINCIPLE:

Serum Pyruvic Oxaloacetate transaminase catalyzes the reaction between alpha - ketoglutaric acid and L-aspartate giving glutamate and oxaloacetate. Oxaloacetate, in the presence of malate dehydrogenase (MDH) reacts with NADH giving malate and NAD. The

rate of NADH decrease is determined photometrically and is directly proportional to the SGOT activity in the sample.

PROCEDURE:

A working reagent was prepared by mixing Reagent I: (Buffer reagent) and Reagent-II: (Enzyme reagent) in the ratio of 4:1 respectively. The working reagent and the sample were mixed as mentioned below.

SAMPLE	100μL
REAGENT	1000μL

They were mixed well and allowed to stand for 1 minute at 37°C. Absorbance decrease per minute was measured during 3 minutes and the Δ A/min was determined.

CALCULATION:

Δ A/min. * 1746 = U/L of SGOT

4.7.1.3 GLUCOSE:

Glucose is a major source of energy for most cells of the body. Insulin facilitates glucose entry into the cells. It is a well-used biological marker of diabetes characterized by hyperglycemia, which is due to inability of producing insulin. Besides diabetes any kind of repair or replication would use a good amount of glucose. So, it is a good marker for cancer progression or tissue repair.

PRINCIPLE:

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts, under catalysis of peroxidase, with phenol and 4-aminophenazone to form a red-violet quinoeimine dye as indicator.

PROCEDURE:

Mix and incubate the standard and samples in the quantity shown below. Incubation is of 5 minutes at 37°C or 15 minutes at room temperature. Incubation is of 5 minutes at 37°C or

15 minutes at room temperature. Measure the absorbance of Sample (AT) and Standard (AS) against Reagent Blank at 505 nm. The colour is stable for 30 minutes at room temperature.

	BLANK	STANDARD	SAMPLE
SAMPLE	-	-	10μL
STANDARD	-	10 μL	-
REAGENT	1000 μL	1000 μL	1000 μL

CALCULATION:

Total Glucose (mg/dl) = (AT/AS) * Concentration of Standard

4.7.2 LIVER GLYCOGEN ESTIMATION

Glycogen is a multi-branched polysaccharide that serves as a form of energy storage in animals. Mostly stored in liver and being the primary function of liver, its levels are utilized as markers for health of liver tissue.

ESTIMATION OF LIVER GLYCOGEN:

The amount of liver glycogen was measured by the method described by Seifter *et al.*, 1950.

PRINCILPE:

Upon alkali hydrolysis glycogen gets released from the liver tissue by heating. Glycogen being a carbohydrate gets dehydrated by concentrated H_2SO_4 to form furfural. Furfural condenses with anthrone reagent to form a blue-green colored complex which is measurable colorimetrically at 620 nm.

Preparation of sample and solution:

After excision from the animal, approximately one gram of tissue was weighed and transferred into previously weighed test-tube filled with 3ml of 30% KOH. The contents of the tubes were reweighed and the actual weight of sample was determined by difference. The

tissue was then digested by heating the tube for 20 minutes in a boiling water bath. This digest was utilized for further estimation of glycogen.

Reagents:

1. 30% potassium hydroxide
2. 95% sulfuric acid: Prepared by adding 1L of best grade concentrated sulfuric acid to 50 ml distilled water and cooling the resultant solution by keeping it in cold water bath.
3. 0.2% freshly prepared anthrone reagent: Prepared by adding 0.2gm of anthrone in 100ml of 95% sulfuric acid.
4. A working glucose standard solution containing 20 μg/ml of glucose.

PROCEDURE:

After the preparation of samples (tissue digests), it was cooled, transferred to 50 ml volumetric flask and diluted to the mark with water. The contents of the flask were thoroughly mixed; a measured portion it, was then further serially diluted with water so as to yield a solution with tissue concentration of 3-30 μg/ml. Five ml aliquots of these dilutions were then transferred to test tubes. To another two test tubes a standard solution of total of 100μg of glucose in five ml of water & 5 ml of distilled water (which constitutes blank) was delivered respectively. While submerged in cold water every test tube was delivered 10 ml of freshly prepared anthrone reagent and the contents were mixed by swirling the tubes. These tubes were then covered and heated for 10 minutes in boiling water bath. Again they were cooled immediately by submerging the tubes in cold water and read against blank in UV-spectrophotometer at 620nm.

CALCULATION:

The concentration of glycogen in the aliquot is calculated using following equation:

Total μg of glycogen in the aliquot = $(100*U)/(1.11*S)$

U= Absorbance of unknown test solution

S= Absorbance of 100 µg of glucose standard

1.11= Factor determined by Morris for the conversion of glucose to glycogen.

4.7.3 LIVER HISTOPATHOLOGICAL ANALYSIS

4.7.3.1 FIXATION AND PROCESSING OF TISSUE

1) The tissues excised from animals were stored in 10% formaldehyde until they got processed for hystopathological analysis.

2) The left lobe of the liver was then kept in following solutions for 2 hours each i.e. 40% formalin, xylene, 60% alcohol, 70% alcohol, 80% alcohol and twice in 100% (Absolute) alcohol.

3) Then tissues were kept in melted paraffin twice before drying. After they were dried, blocks of paraffinized tissues were cut according to the size of tissue.

4) 5µm thick sections of tissues were serially cut on a leica microtome in horizontal plane and mounted on glass slide with help of glycerin solution. The sections were deparaffinized in xylene and downgraded through descending grades of alcohol and finally water.

5) They were then stained with 10% hematoxyline for 3-5 minutes and staining was intensified by placing in running water.

6) These sections were stained with 10% eosin for 2 minutes and quickly passed through ascending grades of alcohol.

7) Finally they are treated with xylene followed by mounting and analyzing under OLYMPUS (trinocular-CX21FS1) microscope with 400X magnification setting.

4.7.3.2 ANALYSIS OF H&E [HEMATOXYLINE AND EOSINE] STAINED SLIDES

The H&E stained slides were analyzed under photo microscope with 400X magnification setting. Liver sections were scored for necrosis using a graded scoring system for multifocal

necrosis. The liver sections were examined for necrotic cells, extent of inflammation, fibrosis, pyknotic nuclei, and hemorrhage. Scoring was as follows:

0, no necrosis;

+1, minimal, defined as only occasional necrotic cells in any lobule;

+2, mild, defined as less than one-third of the lobular structure affected;

+3, moderate, defined as between one-third and two-thirds of the lobular structure affected;

+4, severe, defined as greater than two-thirds of the lobular structure affected.

From each liver section, randomly 10 lobules were chosen and graded as described above. From those 10 values, mode (the value which appears most frequently) of each was found out. And as the groups contains more than one animal mode of all the individual modes was considered final value for expressing extent of necrosis.

4.8 STATISTICAL ANALYSIS

All the values are expressed as mean ± S.E.M. Statistics was applied using Graph pad prism software version 5.0. Statistical significance between vehicle control, CBZ treated and also between different time points of CBZ treated group was tested using one-way ANOVA test followed by tuckey test. Difference was considered to be statistically significant when $p < 0.05$.

5. RESULT

5.1 BIOCHEMICAL PARAMETERS

5.1.1 Serum parameters

5.1.1.1 Serum SGOT levels

To investigate liver injury Serum SGOT level were compared in Vehicle control group and CBZ treated mice during time course of 0 – 96 hrs. Mice administered with vehicle did not cause any elevation of SGOT at any time point. Whereas CBZ treated mice injury was evident as early as 0 hrs (5th day) and increased up to 48 hrs after CBZ administration and thereafter declined gradually. CBZ treated group of 36th & 48th after 5th day of dosing significantly ($P < 0.05$) increased serum SGOT level than vehicle control group of that time point and CBZ treated control group of 0 hrs time point. The levels were significantly ($P < 0.05$) lower at 72 & 96 hrs time point of CBZ treated group. (Table & Figure 5.1)

Table 5.1: Serum SGOT levels in different time points of vehicle control and CBZ treated groups

Groups	Blood Sampling Time (hrs)						
	0	**12**	**24**	**36**	**48**	**72**	**96**
Vehicle Control	77.58 $\pm$1.76	74.72 $\pm$1.05	72.89 $\pm$ 1.10	77.86 $\pm$ 2.92	80.74 $\pm$2.61	71.07 $\pm$1.46	75.97 $\pm$1.16
CBZ Treated	92.81 $\pm$ 3.17	116.86 $\pm$ 5.12	123.05 $\pm$ 2.69$^{\$}$	131.65 $\pm$ 5.45$^{\$}$	180.01 $\pm$7.58$^{\$@}$	117.96 $\pm$ 4.67$^{\#}$	98.44 $\pm$ 4.79$^{\#}$

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

$ Significant ($P < 0.05$) different from 0 hrs CBZ Treated group

@ Significant ($P < 0.05$) different from 36th hrs CBZ Treated group

Significant ($P < 0.05$) different from 48th hrs CBZ Treated group

VC – Vehicle control, CT – CBZ treated

Figure 5.1: Serum SGOT levels in different time points of vehicle control and CBZ treated groups

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

$ Significant (P<0.05) different from 0 hrs CBZ Treated group

@ Significant (P<0.05) different from 36[th]hrs CBZ Treated group

Significant (P<0.05) different from 48[th]hrs CBZ Treated group

VC – Vehicle control, CT – CBZ treated

5.1.1.2 Serum SGPT levels

To investigate liver injury Serum SGPT level were compared in Vehicle control group and CBZ treated mice during time course of 0 – 96 hrs. Mice administered with vehicle did not cause any elevation of SGPT at any time point whereas CBZ treated mice injury was evident early as 0 hrs (5 [th] day) increased up to 48 hrs after CBZ administration and thereafter declined gradually. CBZ treated group of 36th & 48th after 5th day of dosing significantly (P< 0.05) increased serum SGPT level than vehicle control group of that time point and CBZ treated control group of 0 hrs time point. The levels were significantly (P< 0.05) lower at 72 & 96 hrs time point of CBZ treated group. (Table& Figure 5.2)

Table 5.2: Serum SGPT levels in different time points of vehicle control and CBZ treated groups

Groups	Blood Sampling Time (hrs)						
	0	**12**	**24**	**36**	**48**	**72**	**96**
Vehicle Control	36.73 $\pm$1.43	38.42 $\pm$ 1.93	38.95 $\pm$ 1.74	40.92 $\pm$ 1.74	35.60 $\pm$2.67	38.67 $\pm$1.74	36.35 $\pm$1.50
CBZ Treated	59.30 $\pm$ 4.20	77.02 $\pm$4.35	86.53 $\pm$ 2.81*	90.60 $\pm$ 5.51*	118.28 $\pm$ 7.88[*@]	60.87 $\pm$ 3.52[#]	53.76 $\pm$ 2.59[#]

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

* Significant (P<0.05) different from 0 hrs CBZ Treated group

@ Significant (P<0.05) different from 36th hrs CBZ Treated group

Significant (P<0.05) different from 48th hrs CBZ Treated group
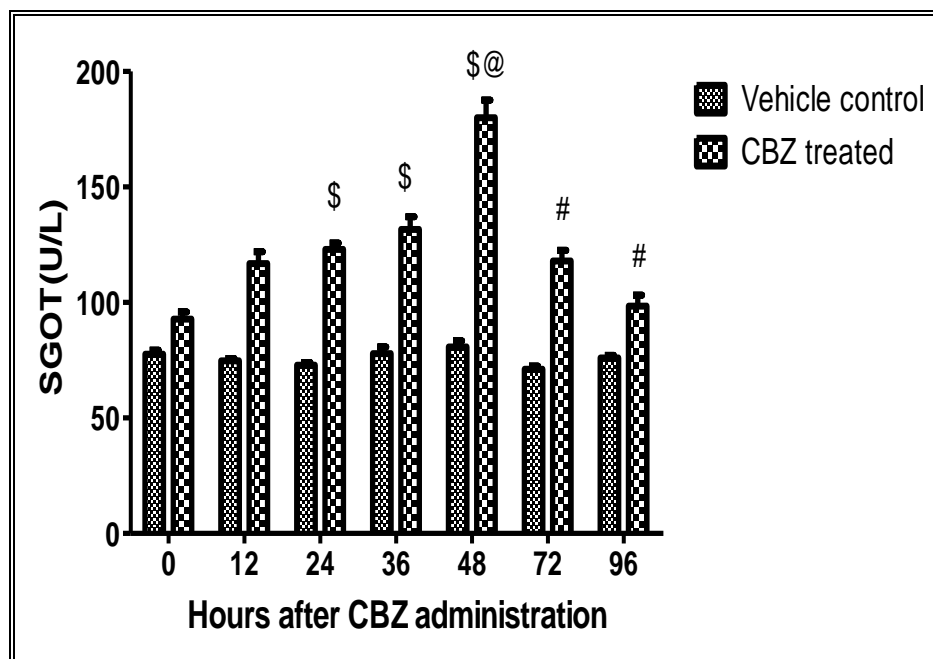
VC – Vehicle control, CT – CBZ treated

Figure 5.2: Serum SGPT levels in different time points of vehicle control and CBZ treated groups

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

* Significant (P<0.05) different from 0 hrs CBZ Treated group

@ Significant (P<0.05) different from 36[th]hrs CBZ Treated group

Significant (P<0.05) different from 48[th]hrs CBZ Treated group

VC – Vehicle control, CT – CBZ treated

5.1.1.3 Serum Glucose levels

To investigate liver injury and tissue repair serum glucose levels were compared in Vehicle control group and CBZ treated mice during time course of 0 – 96 hrs. Mice administered with vehicle did not cause any significant change in glucose at any time point. Whereas in CBZ treated mice elevated glucose level was evident at 0 h (5 th day) and increased thereafter reaching peak at 24hrs after CBZ administration suggesting liver regeneration process demand energy in term of elevated serum glucose level. The proliferation of the cell could be maximum at 36 & 48 hrs as evident from decreased serum glucose level and thereafter glucose stabilized slowly. CBZ treated group of 36hrs after 5th day of dosing significantly (P< 0.05) decreased serum glucose level than vehicle control group of that time point and CBZ treated control group of 0 hrs time point. The levels were significantly (P< 0.05) higher at 48,72 &96hrs time point of CBZ treated group than 36 hrs time point of CBZ treated group. (Table& Figure 5.3)

Table 5.3: Serum Glucose levels in different time points of vehicle control and CBZ treated groups

Groups	Blood Sampling Time (hrs)						
	0	**12**	**24**	**36**	**48**	**72**	**96**
Vehicle Control	120.29 $\pm$1.27	118.77 $\pm$ 1.93	120.49 $\pm$ 2.07	119.6 $\pm$ 2.32	123.47 $\pm$1.13	115.62 $\pm$1.35	119.12 $\pm$1.04
CBZ Treated	133.15 $\pm$ 2.85	160.09 $\pm$2.85	166.92 $\pm$ 5.89	94.45 $\pm$ 2.25$^{*@}$	119.41 $\pm$ 7.69$^{@\#}$	134.66 $\pm$ 8.24$^{@\#}$	129.19 $\pm$ 5.67$^{@\#}$

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

* Significant (P<0.05) different from 0 hrs CBZ Treated group

@ Significant (P<0.05) different from 24[th]hrs CBZ Treated group

Significant (P<0.05) different from 36[th]hrs CBZ Treated group
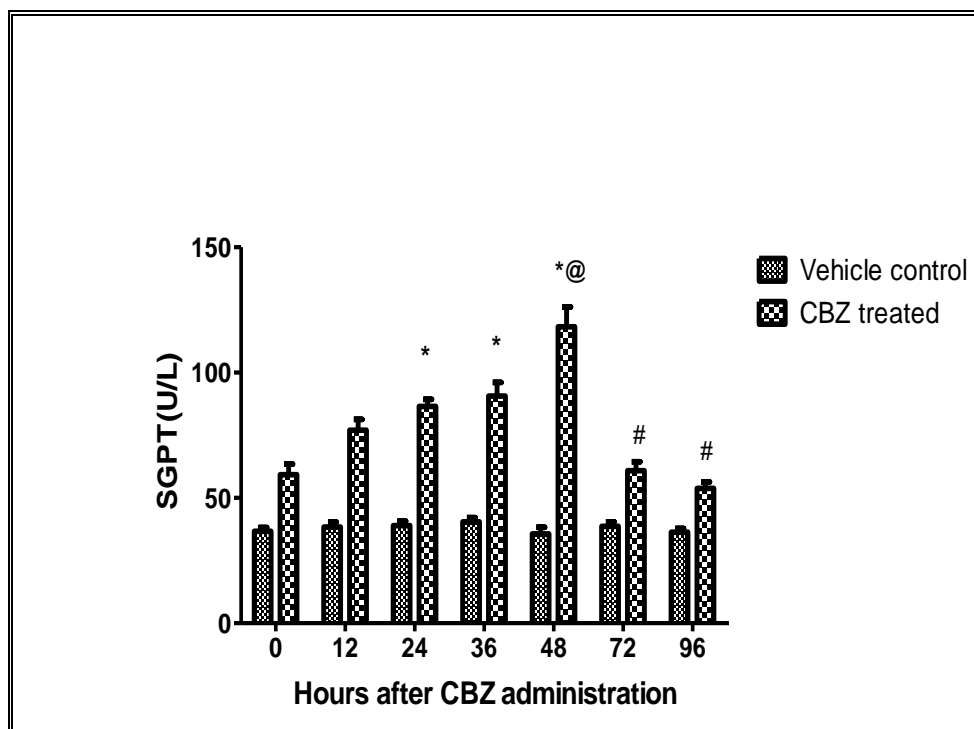
VC – Vehicle control, CT – CBZ treated

Figure 5.3: Serum Glucose levels in different time points of vehicle control and CBZ treated groups

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

* Significant (P<0.05) different from 0 hrs CBZ Treated group

@ Significant (P<0.05) different from 24[th]hrs CBZ Treated group

Significant (P<0.05) different from 36[th]hrs CBZ Treated group

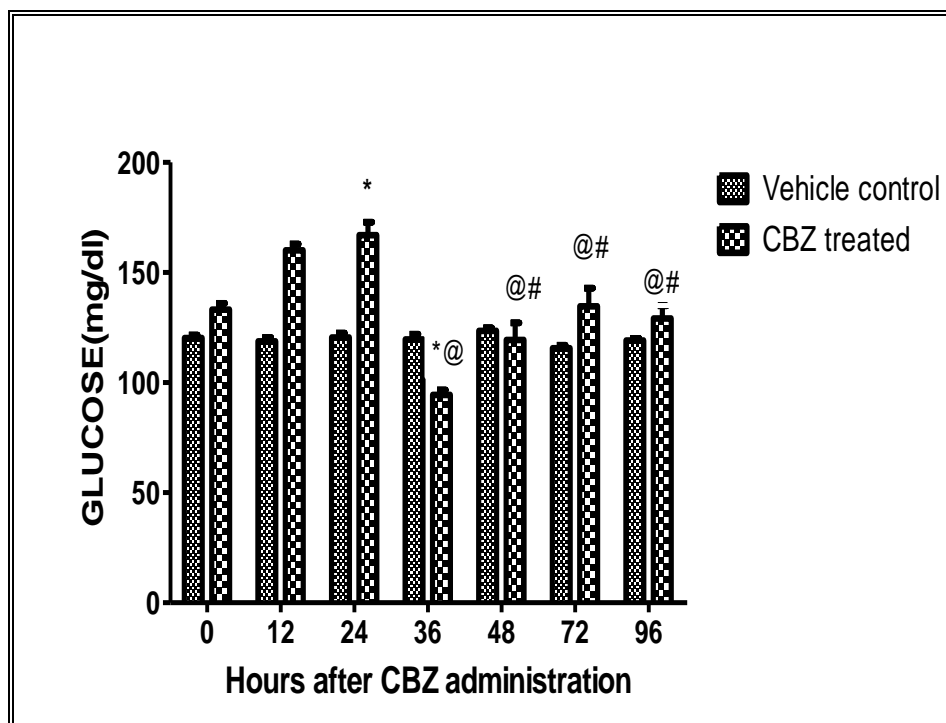VC – Vehicle control, CT – CBZ treated

5.1.2 Tissue parameters

5.1.2.1 Liver glycogen levels

To investigate liver injury and tissue repair (liver regeneration) serum glycogen levels were compared in vehicle control and CBZ treated mice during a time course of 0 – 96 hrs. Mice administered with vehicle did not cause significant change in glycogen level at any time point indicating liver regeneration is normal to maintain homeostasis. Whereas, CBZ treated mice showed declined glycogen levels at 0 hrs (5th Day) and thereafter reaching the lowest level at 48 hrs after CBZ administration. At 48 hrs time point after 5th day of dosing, serum glycogen levels were significantly ($p < 0.05$) lower than vehicle control group of the same time point. This suggests that liver regeneration process requires energy in terms of converting glycogen to glucose for satisfying the demand. The glycogen levels were significantly ($p < 0.05$) higher at 72 and 96 hrs time point of CBZ treated group as compared to 36 and 48 hrs of CBZ treated group suggesting that after 48 hrs. Glycogen levels slowly stabilized indicating regeneration process declined gradually (Table &figure 5.4).

Table 5.4: Liver Glycogen levels in different time points of Vehicle control and CBZ treated groups

Groups	Blood Sampling Time (hrs)						
	0	**12**	**24**	**36**	**48**	**72**	**96**
Vehicle Control	43.06 $\pm$1.07	44.35 $\pm$ 1.84	43.56 $\pm$ 1.49	44.20 $\pm$ 1.26	43.99 $\pm$1.09	43.76 $\pm$1.29	41.78 $\pm$1.72
CBZ Treated	32.49 $\pm$ 1.80	29.32 $\pm$2.59	24.23 $\pm$ 2.38[#]	17.38 $\pm$ 2.22[#]	14.39 $\pm$ 1.72[#]	29.72 $\pm$ 5.34[*@]	35.80 $\pm$ 3.17[*@]

Values are expressed as mean $\pm$ S.E.M. of 5 - 6 mice in each time point.

Significant (P<0.05) different from 0 hrs CBZ Treated group

* Significant (P<0.05) different from 48 hrs CBZ Treated group

@ Significant (P<0.05) different from 36hrs CBZ Treated group

VC – Vehicle control, CT – CBZ treated

Figure 5.4: Tissue Glycogen levels in different time points of Vehicle control and CBZ treated groups

Values are expressed as mean ± S.E.M. of 5 - 6 mice in each time point.

Significant (P<0.05) different from 0 hrs CBZ Treated group

* Significant (P<0.05) different from 48 hrs CBZ Treated group

@ Significant (P<0.05) different from 36hrs CBZ Treated group
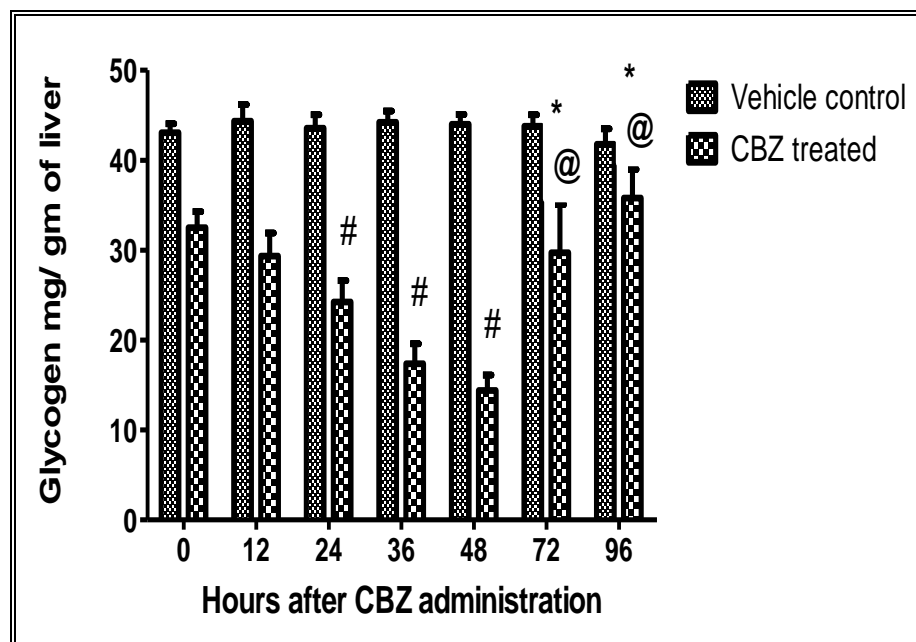
VC – Vehicle control, CT – CBZ treated

5.1.2.2 HISTOPATHOLOGICAL ANALYSIS

In the histopathological study the liver tissue from the vehicle control group showed no signs of necrosis or loss of hepatocytes. In contrast the CBZ treated animals showed a pattern of remarkable hepatic necrosis and loss of hepatocytes, especially around the central vein. We examined the liver sections stained by H&E for necrotic cells, extent of inflammation, fibrosis, and pyknotic nuclei. At 0 hrs, very little portion of the liver paranchyma showed necrosis i.e. only occasional necrotic cells in any lobule. More distinct evidences of necrosis were visible at 24 hrs with more number of pyknotic nuclei. The necrosed cells were concentrated around the central vein, but cells around the portal area were unaffected. Extensive centrilobular necrosis started at 36 hrs with infiltration of inflammatory cells and cell death. In the centrilobular area, liver-cell columns were broken up; the individual cells were separated from each other and appeared to be crumbling, indicating degeneration. Necrosis was very prominent at 48 hrs and fine vacuoles appeared. Between 36 and 48 hrs, the periportal cells in the CT group also started showing occasional mitosis, in sharp contrast to increased widespread necrosis approaching the periportal area. However, presence of probable cell division and proliferation activity afterwards appeared to restrain the progression of injury at 72 and 96 hrs leading to recovery of animals from CBZ induced hepatotoxicity. At 72 and 96 hrs presence of apoptotic bodies was also evident suggesting probable termination process of regeneration (Figure and Table: 5.5).

Table 5.5: Histopathological scoring for different time points of vehicle control and CBZ treated groups

The scoring of the extent of necrosis was as below,

Groups	Liver collection time (hrs)						
	0	**12**	**24**	**36**	**48**	**72**	**96**
Vehicle Control	0	0	0	0	0	0	0
CBZ Treated	+1	+1	+2	+2	+3	+2	+2

Liver sections were scored for necrosis using a graded scoring system for multifocal necrosis & the values are expressed as:

0: no necrosis

+1:Minimal, defined as only occasional necrotic cells in any lobule,

+2:Mild, defined as less than one-third of the lobular structure affected,

+3:Moderate, defined as between one-third and two-thirds of the lobular structure affected,

+4: severe, defined as greater than two-thirds of the lobular structure affected.

VC – Vehicle control, CT – CBZ treated
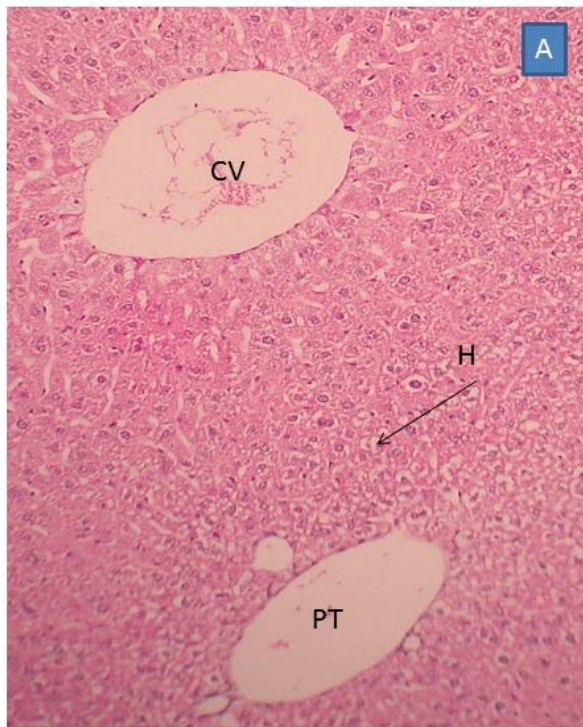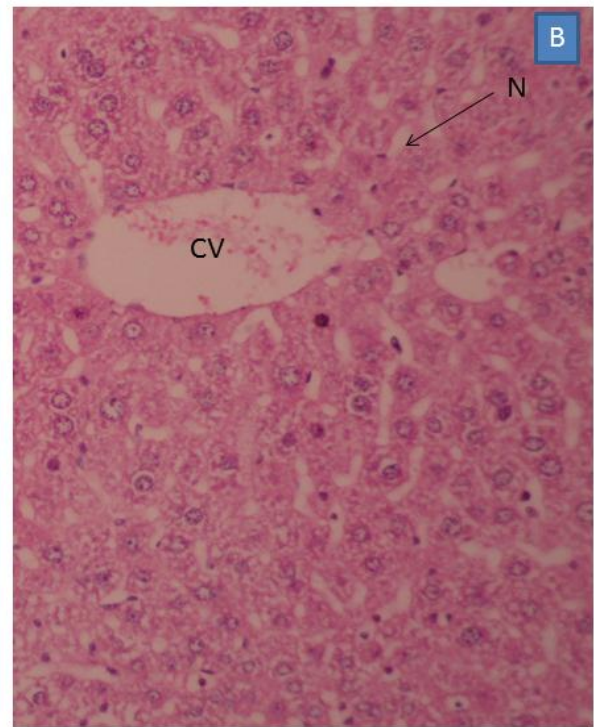
Vehicle Control	0 hrs After CBZ treatment
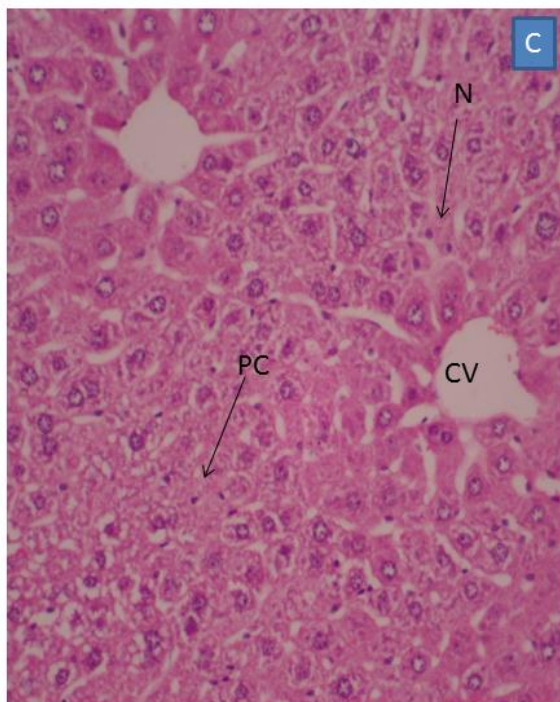
12 hrs After CBZ treatment	24 hrs After CBZ treatment
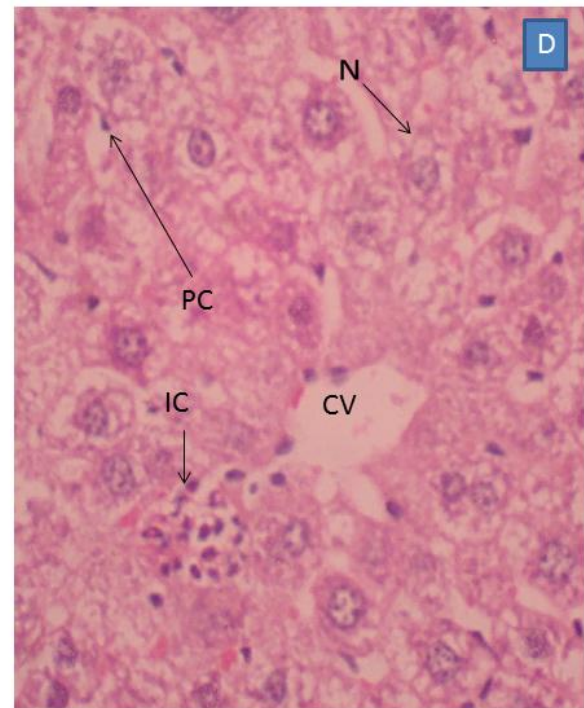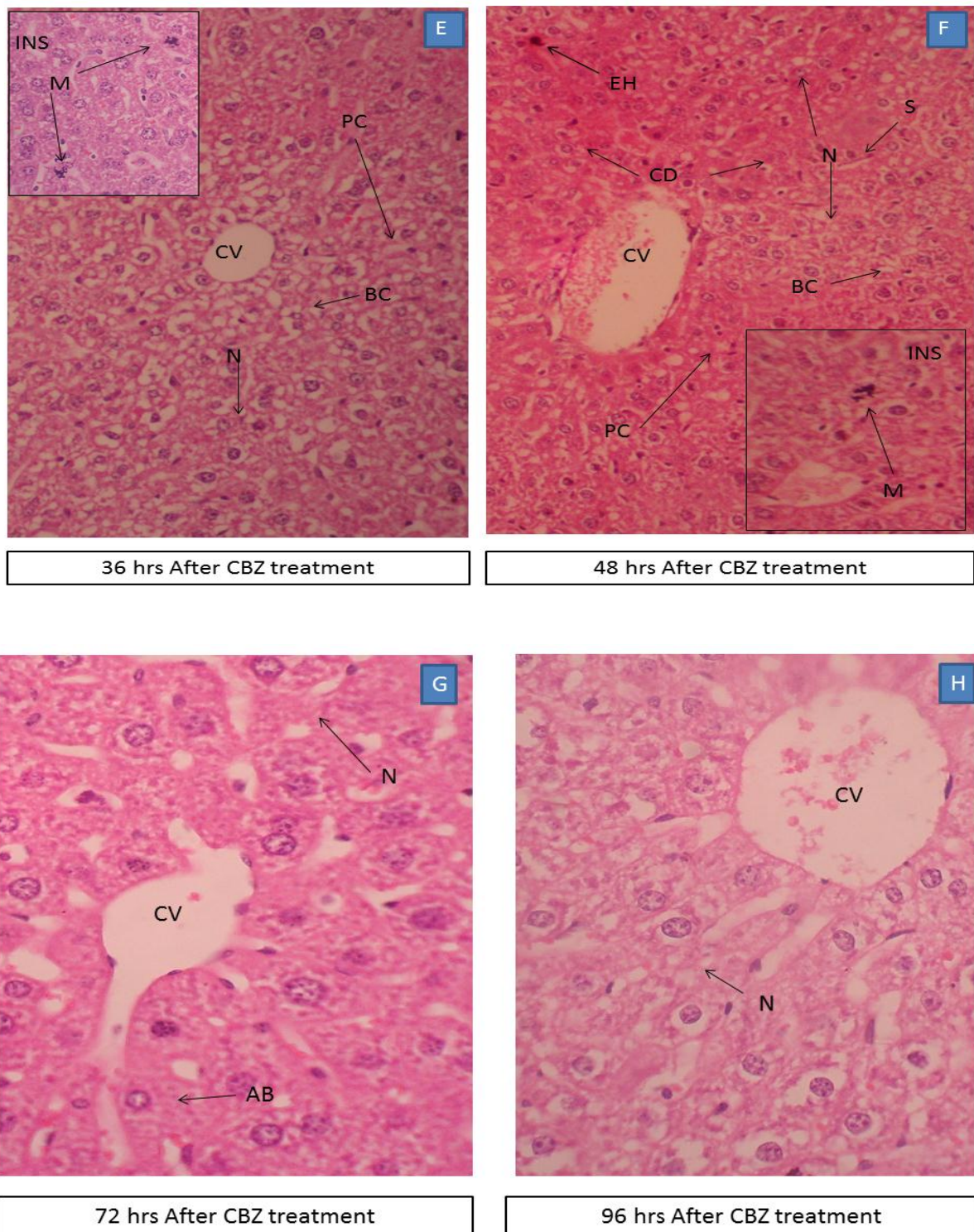
Figure 5.5: Histopathological analysis of vehicle control and CBZ treated mice liver
Representative photomicrographs of H&E stained liver sections of vehicle control group and CBZ treated group.

A) Vehicle control (0 hrs): Vehicle control groups of all the time points showed similar liver archirstructure. Hence, for simplicity we included only one slide. B) CBZ treated – 0 hrs. C) CBZ treated – 12 hrs. D) CBZ treated – 24 hrs. E) CBZ treated – 36 hrs: Separate insert shows mitotic cells. F) CBZ treated – 48 hrs: Time of maximum liver injury: Separate insert shows mitotic cells G) CBZ treated – 72 hrs. H) CBZ treated – 96 hrs: Time near normalization. Original magnification, 400X. CV – Central vein, PT – Portal triad, AB – Apoptotic bodies, N – Area of necrosis, M – Mitotic cells, EH – Eosinophilic hepatocytes, IN – Inflammatory cells, PC - Pyknotic nuclei cell, CD - Cell death, S – Enlarged sinusoidal space, BC – Broken hepatocyte

6. DISCUSSION

Liver is an interesting organ with high regenerative capacity and complex functions (Michalopoulos and DeFrances, 1997; Taub, 2004; Michalopoulos and Khan, 2005; Fausto et al., 2006). Liver receives all exiting circulation from the small and most of the large intestine, as well as spleen and pancreas, through the portal vein (Michalopoulos and DeFrances., 1997). The liver composed of main two types of cell i.e. parenchymal cell and non-parenchymal cell. The parenchymal cell composed of the hepatocyte which is comprised of 60 % of total liver cell population. The non-parenchymal cell includes Kupffer cell, endothelial cell, and ito cell. The hepatocyte regulates the carbohydrate and lipid metabolism. The gluconeogenesis and glycogenolysis are simultaneous process modulated and regulated by the liver. The hepatocyte is the main regulatory body for defense against hepatotoxins and the toxic substance (Tso and McGill et al., 2002).

The fascinating property of liver is to regenerate after chemical insult. The auto protection phenomena of the liver are well reported on the exposure of dose dependent toxicity profile of chemicals (Mehandale et al., 1998). The regenerative capacity of the liver is well known, and the mechanisms that regulate this process have been extensively studied using experimental model systems including surgical resection and hepatotoxins exposure. In classic chemically induced toxicity studies only toxic injury has been measured as the end point of the mechanisms that inflict injury. In addition to toxic response, however, tissue repairs, a simultaneous biologic compensatory response that accompanies chemical-induced injury (Mehandale et al., 1991).

Several studies suggest that the rate and extent of tissue repair as a response to the injury inflicted by toxicants determines the ultimate outcome of hepatotoxicity (Chandaet al., 1996; Mehendale at al., 1989). Blockage ofthe tissue repair leads to progression of injury, led to hepatic failure and death (Mangipudi et al., 1996). Because stimulation of tissue repair is a biologic response that accompanies injury, quantifying this response in addition to measuring injury might be helpful in predictive toxicology (Mehandale et al., 1995).Several studies proposed that injury and tissue repair are simultaneous but opposing parallel responses to administration of toxic chemicals. Stimulated cell division and tissue repair by exposure to

toxicants has several implications. Clearly, the newly divided cells are available to restore tissue structure and function (Mehandale et al., 1991).

Drugs can cause a diverse array of liver injury, which may be acute or chronic. Adaptation to injurious effects, with only a transient increase in liver function, can occur. Drug induced liver injury is a result of the drug overdose or idiosyncratic reaction (Kaplowitz, 2004).

Carbamazepine is widely used antiepileptic drug causes liver injury due to idiosyncratic reaction (Pallock., 1987; Kaufman and Shapiro., 2000; Björnsson andOlsson; 2005).CBZ is extensively metabolized in humans to over 30 metabolites, and the most important pathway involves the formation of the stable CBZ-10, 11-epoxide followed by hydroxylation to CBZ-10,11-*trans*-dihydroxy (diOH) (Lertratanangkoon and Horning.,1982; Lu and Uetrecht., 2008). The CBZ 2.3- epoxide, CBZ 10.11- epoxide and 3- OH CBZ metabolite are responsible for the CBZ induced liver injury. The CBZ causes hepatocellular injury in hepatic lobular structure around central vein.

The CBZ induced liver injury model has recently established and the tissue repair mechanism is not studied yet. So our interest was to test whether the Female Swiss albino mice were also susceptible for CBZ induced liver injury. we developed liver injury in mice by using regimen previously reported dose (Higuchi et al., 2012). Dose optimization were been carried out to accesses for the model stability and mortality. The Swiss albino was given CBZ (400mg/kg for 4 days and 800 mg/ kg on 5th day) result in the 50% mortality. It suggesting that the Swiss albino mice are more sensitive toward the CBZ induced liver injury. The stability of the model was not observed due to high mortality rate. So the reduction of the initial dose had been carried out to reduce the mortality rate in Swiss albino mice model. The Swiss albino mice were given CBZ (350mg/kg for 4 days and 800 mg/ kg on 5th day) dosing regimen show better model stability and less mortality rate throughout the treatment period. Based upon the theory of autoprotection (Mehendale, 1994), we decided to give initial lower dose (350mg/kg for 4 days) followed by higher dose (800mg/kg on 5th day). The animal was survived after the dose of the 800mg/kg. So the dose regime was chosen for the study of CBZ induced liver injury in Swiss albino mice.

The serum SGOT and SGPT are well known liver injury markers. The disturbance in the transport function of the hepatocyte as a result of hepatic injury causes the leakage of enzyme from the cell due to altered permeability of the membrane results into the decrease the level of enzyme in hepatocyte cell and raised level in serum (Zimmerman and Seefe., 1970; Plaa, 1986; Yadav et al., 2010). The increased in levels of SGOT and SGPT due to the hepatic injury was reported in chemical induced liver injury (Yadav et al., 2010).

The generation of the ROS involved in drug-induced liver injury via mitochondrial dysfunction or hepatocyte necrosis (Jaeschke et al., 2002). The CBZ causes the elevated level of the liver marker due to the cellular mediated injury via ROS production and release of the inflammatory mediator i.e. chemokine and cytokine. The overproduction of free radicals was the initial step in a chain of events that eventually leads to membrane lipid peroxidation and ultimately to cellular apoptosis and necrosis (Basu, 2003). The initially elevated level of the Serum SGPT and SGOT level up to 48 hrs suggested that the tissue injury is observed maximum at this point and mediated by the primary and secondary inflammation. The primary inflammation is occurred due to the CBZ and its metabolite i.e. CBZ 10, 11 epoxide and after that the secondary inflammation induced by the stimulation inflammation due to the necropsied hepatocyte which activate further through release inflammatory mediator (Higuchi et al., 2012). The elevated injury after the elimination of CBZ and its metabolite observed due to the secondary inflammation (Higuchi et al., 2012). The decline in the levels of the Serum level of the SGPT and SGOT indicate that the tissue repair has been simultaneous accompanied with tissue injury. The level of the SGOT and SGPT were higher in CBZ treated group as compared to vehicle treated group, suggesting that the CBZ causes stage II model of toxicity.

The liver having complex structure comprised of sinusoidal space, hepatocyte, Kupffer cell, central vein, portal triad composed of the bile duct, hepatic artery, and portal vein. The tissue injury led to change in the functional structure of the liver tissue. After chemical insult the abnormality in the liver structure was observed due to injury. The centrilobular necrosis was characteristic feature of CBZ induced liver injury model. The extent of necrosis was scored by the grading system. The extent of necrosis was observed in initial time point 0 – 48

hrs indicate that the tissue injury was observed. In the initial time point, the hepatocyte damage, presence of pyknotic nuclei, cell death was observed due to the ROS production and release of the cytokine and chemokine. The damage of hepatocytes was higher at 48 hrs suggesting that CBZ injury were persists after the elimination of the drug.

The presence of mitotic cell was observed in Carbon tetrachloride induced liver injury in rat that suggested the tissue repair response after the liver injury (Ebaid et al., 2013). The extent of necrosis and presence of the pyknotic nuclei up to 48 hrs suggested maximum injury at this time point. Simultaneous the presence of occasional mitotic cell were also observed which indicate that the tissue repair has been also occurred which was visualized under the H&E staining at 400X magnification. The presence of the mitotic cell reflects the cell proliferation at 36 hrs which also observed at the 48 hrs time point. This process suggested that the tissue repair occurs after toxic insult induced liver injury (Mehandale et al., 1991). The group treated with vehicle show no destruction of the liver architecture. The presence of apoptotic body at the 72 & 96 hrs time point suggesting that the cessation of the liver regeneration was occurred. This histopathological data support the biochemical findings.

The Serum glucose level is regulated by liver. After chemical insult the change in the serum glucose level reflect the liver injury. The glucose is stored in the form of the glycogen in the hepatocyte. When the glucose level is observed low in the blood, liver tend to release the glucose by glycogenolysis process. The liver regeneration requires energy for the synthesis of new cell development. The elevated level of the glucose up to the 24 hrs reflect that the liver regeneration require energy. The decrease in the level of the glucose reflects maximum cell proliferation at that time point suggesting the tissue repair response (Kodavantiet al., 1990; Soni and Mehendale et al., 1994; Anand and Mehendale et al., 2004).

The liver glycogen level reflects the energy demand of body. Hepatocyte damage lead to the change in the level of the hepatic glycogen content which reflects chemical induced injury (Chanda and Mehendale et al., 1995; Chanda and Mehendale et al., 1996). In the CBZ induced liver injury, levels of glycogen up to 48 hrs decreases gradually. Whereas the vehicle control group did not show any change in the level of the glycogen. The decreased level of the

glycogen indicates the tissue repair response to toxic insult. The glycogen level was decreased gradually to meet the energy requirement in the term of the elevated serum glucose level. Gradual decrease in the glycogen level suggesting the tissue repair response in CBZ treated mice.

7. Conclusions

In conclusion, female Swiss albino mice are susceptible to CBZ induced liver injury. This finding suggested that the tissue injury was observed due to the toxic dose of the CBZ. Whereas tissue repair response play an important role in the reversal of CBZ induced liver injury. The elevated level of liver injury markers and histopathological data suggest the liver injury severity, while the presence of the mitotic body, decline of the liver injury markers and consumption of energy substrate indicate that tissue repair has been simultaneously accompanied to regress the liver injury induced by CBZ in female Swiss albino mice.

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APPENDIX

LIST OF INSTRUMENT

INSTRUMENT	SOURCE
Prietest TOUCH Biochemistry Analyzer	2.622A, ROBONIK Pvt. Ltd.
UV Spectrophotometer	SCHIMADZU- 1800
Centrifuge	Remi motor Pvt. Ltd.
Microscope	ALMICRO- micro measure &instrument- BM-6 mo
Electronic Balance	Lab series C-3, Roy electronics
Micropipette	Accupette
Hot air Oven	Elico Ltd.
Microtips&Eppendorfs	Tarson Ltd.
Aluminium foils	Nice Chemical Ltd.

LIST OF CHEMICAL

CHEMICAL	SOURCE
Carbamazepine	Swati chemical Ltd.
Diagnostic Kits	Lab care Diagnostic Pvt. Ltd.

All other Lab chemicals were purchased from CDH Ltd. New Delhi.

Mouse diet was procured from Pranav agro, Pune.