2. Literature review

2.1 Drug induced liver toxicity

The liver is the central organ for the metabolism of every foreign substance. Mostly all drugs are lipophilic. In hepatocytes drugs are rendered hydrophilic to yield water soluble products by biochemical processes which can be excreted in urine or bile. This process is called as biotransformation. It consists of phase 1 and phase 2 reactions. In the phase 1 reaction, oxidation or demethylation occurs. These reactions are mediated by cytochrome P450. A variety of oxidative phase 1 reactions are performed by the enzymes that make up the P450 system. Typical phase 1 reaction will generate a hydroxyl group, which can then participate in phase 2 reactions. In phase 2 reactions, a large; water soluble polar group is introduced to hydroxyl oxygen by glucoronidation and or sulfation. Another metabolic pathway for detoxifying many compounds involves glutathione, a thiol-containing tripeptide, capable of binding to potentially harmful electrophilic compounds through glutathione S-transferase. This reaction is central to the detoxification of a number of compounds such as acetoaminophen (Lee., 2003)

2.1.1 Types of drug reactions

2.1.1.A) Direct Toxic Reactions

Acetaminophen is an example of an agent that causes a direct toxic reaction. It is used as a non narcotic pain reliever. The metabolic pathway for acetaminophen involves phase 1 and 2 reactions, glutathione detoxification, and the formation of reactive intermediates, which disrupt cell macromolecules. Through CYP 450 an electrophilic compound, N-acetyl-p-benzoquinoneimine (NAPQI), is formed which can bind to covalently to cell macromolecules, thereby disrupting mitochondrial and possibly nuclear function. The formation of covalent bonds is prevented if NAPQI can be detoxified by conjugation (through glutathione-s-transferase) to generate, mercapturic acid, a harmless, water-soluble product excreted by the kidney. Depletion of glutathione reduces this last defense against the formation of NAPQI-related intracellular adducts. Thus, any situation that leads to the depletion of glutathione will increase toxicity, where as an increase in available glutathione stores will diminish this toxicity. Starvation and alcohol deplete mitochondrial glutathione, whereas N-acetylcysteine replenishes glutathione stores and protects against acetaminophen induced injury. Enzyme which is responsible for the conversion of acetaminophen to NAPQI, is induced by ethanol and inhibited by cimetidine.
Thus, at several metabolic stages, ethanol increases toxicity, whereas cimetidine may serve as an antidote.

2.1.1.B) Idiosyncratic Reactions

The majority of drug-related reactions, such as those observed with isoniazid, are idiosyncratic and unpredictable. Isoniazid is used for prophylaxis against the tuberculosis. Several factors explain the relatively common toxic reactions observed. First, the simultaneous use of alcohol or rifampin may augment the toxicity of isoniazid. Second, elderly persons may be more likely to have toxic reactions than younger persons. Third, genetic differences also play an important role, since persons who are capable of rapid acetylation of isoniazid have an increased likelihood of toxic reactions resulting from the formation of acetylhydrazine, which is then transformed by cytochrome P450 into a reactive metabolite. Some studies suggest that persons with slow acetylation are at greater risk for a toxic reaction through a separate pathway that leads to the formation of hydrazine, which itself may be toxic.

2.1.1.C) Combined Toxic and Allergic Reactions

A halothane is a seldom-used anesthetic agent that can induce a combination of toxic and allergic reactions leading to liver injury. Severe halothane-related hepatitis generally develops after multiple exposures to the drug such as those that can occur on subspecialty surgical services. Protein adducts formed from the initial toxic reaction provide the hapten for the formation of antibodies, so that with subsequent exposure, antibody and cellular recognition of the halothane–protein-adduct antigen on the hepatocyte surface leads to cell lysis.

2.1.1.D) Allergic Hepatitis

Drugs such as phenytoin can cause a systemic allergic reaction characterized by fever, rash, lymphadenopathy, eosinophilia, and the presence of eosinophils or granulomas in liver-biopsy specimens. This allergic reaction is accompanied by both hepatocyte necrosis and cholestasis. The mechanisms responsible for the combined allergic and hepatotoxic reactions are not completely clear.

2.1.1.E) Cholestatic Reactions

The drugs that mainly affect bile flow, causing cholestatic injury, include estradiol, chlorpromazine, trimethoprim–sulfamethoxazole, rifampin, erythromycin estolate, nafcillin, and captopril. The mechanism of cholestatic injury remains unclear. Estradiol and other estrogens
have been shown to decrease bile flow and $\text{Na}^+$/K$^+$-ATPase, change tight junctions between cells, and alter the fluidity of the hepatocyte membrane.

**2.1.1.F) Drug-Induced Chronic Hepatitis**

Methyldopa and a number of other compounds have been found to cause liver damage that closely resembles autoimmune chronic active hepatitis. The classic agent producing this reaction is oxyphenisatin, a laxative that has been withdrawn from the market. Early identification of such drug-related chronic hepatitis is not easy; cirrhosis may develop before the hepatitis is diagnosed. Apart from this, identifying the drug or toxin that has caused the cirrhosis is difficult retrospectively if the patient has been consuming alcohol or if unrecognized viral hepatitis is present.

**2.1.2 Mechanism of cellular injury**

Injury to liver cells occurs in various patterns specific to the intracellular organelles affected. The normal hepatocytes can be injured in various manners such as

- High-energy reactions involving cytochrome P-450 enzymes lead to covalent binding of drug to intracellular proteins, producing intracellular dysfunction resulting in the loss of ionic gradients, a decline in ATP levels, and actin disruption, cell swelling, and cell rupture.
- Drugs that affect transport proteins at the canalicular membrane can interrupt bile flow. Certain drugs bind to or disable the bile salt export protein. This process causes cholestasis.
- Disruption of intracellular calcium homeostasis leads to the disassembly of actin fibrils at the surface of the hepatocyte, resulting in blebbing of the cell membrane, rupture, and cell lysis.
- Drugs are relatively small molecules and, therefore, rarely evoke an immune response. However, biotransformation involving high-energy reactions can result in the formation of adducts that is, drugs covalently bound to enzymes. Adducts that are large enough to serve as immune targets may migrate to the surface of the hepatocyte, where they can induce the formation of antibodies (antibody mediated cytotoxicity) or induce direct cytolytic T-cell responses . The secondary cytokine response thus evoked may cause inflammation and additional neutrophil-mediated hepatotoxicity.
• Programmed cell death (apoptosis) can occur along with immune-mediated injury. This can destroy hepatocytes through tumor necrosis factor (TNF) and the Fas pathways, with cell shrinkage and fragmentation of nuclear chromatin. Proapoptotic receptor enzymes, if activated by drugs, will compete with survival pathways within the cell, and this dynamic interaction may shift the balance either in favor of or against further cell damage.

• Still other pathways to injury may develop when drugs damage to mitochondria, disrupting fatty-acid oxidation and energy production. When drugs bind to respiratory-chain enzymes or mitochondrial DNA, oxidative stress results, with ensuing anaerobic metabolism, lactic acidosis, and triglyceride accumulation.

• Other cells within the liver may be the target of drug injury or serve as modulators of other reactions. For example, Kupffer’s cells activate cytokines that may amplify injury, and fat-storage cells (stellate cells) or macrophages may augment injury, produce fibrosis, or form granulomas.

2.2 Selected liver toxicants & drugs

2.2.1 Carbon tetra chloride (CCl₄)

Liver injuries induced by CCl₄ are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs (Lin et al., 2002 & Clawson, 1989). It has been established that CCl₄ is accumulated in hepatic cells and metabolically activated by cytochrome P450 dependent monooxygenases to form a trichloromethyl radical (CCl₃). This radical can either covalently bind to a variety of hepatic macromolecules (Gregory, 1966 & Reynolds, 1967) or initiate lipid peroxidation (Slater, 1966). Either of these processes could lead to the denaturation of critical metabolic pathways with ensuing toxicity. This model is supported by a variety of studies, including investigations demonstrating in vivo covalent binding of radiolabeled CCl₄, to cellular macromolecules (Reynolds, 1967), alterations in the toxicity of CCl₄ and manipulations that are known to affect the activity of the cytochromes P-450 pathways (Marchand et al., 1970). Further reconstitution studies with purified cytochromes P-450 have confirmed their role in the reduction of CCl₄, (Noguchi et al., 1982).

Juhad et al., 1965 were the first to suggest that cell death from CCl₄, was due to alterations in Ca²⁺ metabolism. Increase in cytosolic free [Ca²⁺] leads to the activation of a variety of
hydrolytic enzymes and ultimately leads to cell death. Further evidence has indicated that the cytochrome P-450 system and Ca\(^{2+}\) pump are in close proximity to each other on the microsomal membrane. This proximity could facilitate the toxic process. These studies suggest that damage to the ATP-dependent microsomal Ca\(^{2+}\) pump may be the critical event in initiating CCl\(_4\) toxicity. Since the above mechanisms will have a direct or indirect effect on mitochondria, we have extended our investigations to study the expression of genes related to mitochondrial changes and their molecular mechanisms before and after CCl\(_4\) intoxication.

### 2.2.2 D-Galactosamine (D GalN)

D-galactosamine is a well established hepatotoxicant induces a diffuse type of liver injury closely resembling human viral hepatitis (Decker and Keppler, 1972). A single injection with d-galactosamine can decrease the uracil nucleotides in the liver and heart (Keppler and Decker, 1969; Olivares and Rossi, 1987). Galactosamine markedly depletes hepatic UDP glucuronic acid (UDP-GA) whereas extrahepatic UDP-GA is minimally affected. This suggests that GAL predominantly inhibits hepatic glucuronidation. It disrupts the synthesis of essential uridylate nucleotides resulting in organelle injury. Depletion of these nucleotides ultimately impairs the synthesis of protein and glycoprotein, leads to progressive damage of cellular membranes resulting in a change in permeability of the cellular membrane which leads to enzyme leakage from the cells (Keppler et al., 1970; Abdul_hussain and Mehendale, 1991).

Although D-GalN has been known as a hepatotoxin causing necrosis, it has also been reported to induce apoptosis in the liver of rats (Sun et al., 2003; Tsutsu et al., 1997a). As it is known, DNA fragmentation is an indicator of apoptotic cell death (Penning et al., 1998; Tsutsui et al., 1997b). The high incidence of apoptosis in D-GalN intoxication was explained on the basis that toxicity of D-GalN is mediated through tumor necrosis factor (TNF \(\alpha\), which causes apoptosis in liver cell (Itokazu et al., 1999; Muntane et al., 1998; Wang et al., 2007). TNF \(\alpha\) is synthesized in the Kupffer cells and may be responsible for induction of apoptotic and necrotic cell death of hepatocytes (Molina and Abumrad, 2000). While there is a lot of information on intracellular effects induced by TNF \(\alpha\), its mechanism of cytotoxicity is still unknown. Several studies indicate that oxygen radicals may mediate some of the effects of TNF \(\alpha\) (Adamson and Billings, 1992; Camussi et al., 1991). Taken together these observations, we included in our
study whether D-GalN could induce oxidative stress and necrosis/or apoptosis via intrinsic pathway i.e. (involving mitochondrial damage).

### 2.2.3 Alcohol

Alcohol is a direct systemic toxin although it is widely consumed in the world. Chronic alcohol consumption leads to several metabolic disorders including hepatic and extra hepatic diseases (Lieber, 2000). The first step in the metabolism of alcohol is the oxidation of alcohol to acetaldehyde catalyzed by alcohol/dehydrogenase containing the coenzyme NAD$^+$. The acetaldehyde is further oxidized to acetic acid and finally CO$_2$ and water through the citric acid cycle. A number of metabolic effects from alcohol are directly linked to the production of an excess of both NADH and acetaldehyde.

A central role in the toxicity of alcohol may be played by acetaldehyde itself. Although the liver converts acetaldehyde into acetic acid, it reaches a saturation point where some of it escapes into the blood stream. The accumulated acetaldehyde exerts its toxic effects by inhibiting the mitochondria reactions and functions. A high acetaldehyde level impairs mitochondria function; metabolism of acetaldehyde to acetic acid decreases, more acetaldehyde accumulates, and causes further liver damage.

Various studies have proved that alcohol is metabolized in the liver mainly by cytochrome P450 2E1 into various reactive oxygen species (ROS), e.g. 1-hydroxyethyl radical, hydroxyl radical, and superoxide radical (Cederbaum et al., 2001; Abrahman Et al., 2002). These harmful species are known to cause oxidative degeneration of cellular molecules, which results in cell injury and dysfunction in liver and other organs. (Cederbaum et al., 2001; Abrahman et al., 2002; Purohit et al., 2003).

Investigations have suggested that alcohol causes direct damage to the liver mitochondrial membrane (Adachi et al., 2004). Hence in our studies we have determined the effect of alcohol on the expression of genes related to mitochondrial damage and induction of cell death.
2.2.4 Paracetamol (Acetaminophen)

Paracetamol is widely used as an analgesic and antipyretic agent, but it can produce severe hepatic injury when an overdose occurs. In therapeutic doses, about 80% of paracetamol is conjugated directly and forms sulfate and glucuronide esters before oxidation, and these conjugated esters are excreted in bile or urine. However, a small amount of paracetamol is converted by hepatic cytochrome P450 (CYP2E1) to a highly reactive and toxic quinone intermediate, N-acetyl-para-benzo-quinoneimine (NAPQI) (Mitchell et al., 1974; Miner and Kissinger, 1979; Dahlin et al., 1984; Holme et al., 1984). This intermediate (NAPQI) is known to bind covalently to intracellular macromolecules, deplete glutathione, cause oxidative stress, and alter calcium and/or thiol status in liver cells, all leading to hepatocellular injury. In toxic doses, paracetamol causes acute centrilobular hepatic necrosis with collapse of the reticulin framework (Clark et al., 1973b; James et al., 1975).

It is generally accepted that the ultimate form of hepatic damage caused by paracetamol is necrosis (Adams et al., 2001; Pierce et al., 2002; Knight and Jaeschke 2002; Gujral et al., 2002). However, several reports have presented evidence for the occurrence of apoptosis in paracetamol-induced hepatic damage. For instance, Ferret and co-workers have shown that caspase-3 and 9 activities were slightly increased in mice that were administered an hepatotoxic dose of paracetamol (Ferret et al., 2001), possibility of direct induction of apoptosis by the cytotoxic metabolite of paracetamol. N-acetyl-p-benzoquinone imine, additional factors, cytokines such as tumor necrosis factor and CD95 ligand have been implicated in paracetamol induced liver damage (Laskin et al., 1995; Zhang et al., 2000; Gardner et al., 2002; Fiorucci et al., 2002). Considering the above facts, paracetamol was selected to screen its effect on mitochondrial damage leading to apoptosis or necrosis.

2.2.5 Isoniazid (INH), Rifampicin (RIF) and Pyrazinamide (PYZ)

Anti-tubercular (AT) drugs are the commonest agents causing serious, clinically significant drug induced liver disease in the developing countries (Acharya et al., 1996; Hwang et al., 1997). Most commonly used AT drugs like Isoniazid (INH), Rifampicin (RIF) and Pyrazinamide (PYZ)
are hepatotoxic. Various factors predisposing to ATD hepatotoxicity, both genetic and acquired, are well delineated (Huang et al., 2002; Huang et al., 2003). The hepatotoxicity of INH is thought to be initiated by cytochrome P450 (CYP) mediated metabolism of INH to acetylhydrazine and hydrazine (Sarich et al., 1999; Sarich et al., 1996). RIF, which is generally co-administered with INH in the treatment of tuberculosis, enhances hydrazine production (Pessayre et al., 1977) by enzyme induction. The high reactivity of hydrazine with sulphydryl groups results in glutathione depletion within the hepatocytes (Sarich et al., 1999) leading to cell death (Macho et al., 1997; Van den Dobbelsteen et al., 1996).

A few experimental studies have also demonstrated the critical role of glutathione in AT drugs induced hepatotoxicity (Attri et al., 2000; Sodhi et al., 1997). But only depletion of hepatic glutathione by INH or its metabolites, could not explain the AT drug induced hepatotoxicity.

Mitochondrion is an important organelle for cell survival and functions. Mitochondrial dysfunctions have been observed in diclofenac hepatotoxicity. Mitochondrial permeability transition (MPT) is focused as a mechanism for drug induced liver cell injury (Masubuchi et al., 2002; Burcham and Harman, 1991), causing both necrotic and apoptotic cell death. INH could produce apoptosis in Hep G2 and AHH1 cell line (Schwab and Tuschl, 2003) as well as necrosis in rabbit liver (Sarich et al., 1996). However, there is lack of experimental data that supports the role of mitochondria in AT drugs induced liver injury. In the present study, we have investigated the possible role of mitochondria, particularly oxidative stress and involvement of MPT in AT drugs induced liver cell injury.

### 2.3 Selected hepatoprotective agents

There are numerous plants and polyherbal formulations claimed to have hepatoprotective activity (Vijayan et al., 2003; Oh et al., 2004; Gilani et al., 2005). Nearly 150 phytoconstituents from 101 plants have been claimed to possess liver protecting activity (Doreswamy and Sharma, 1995), but only some of them are still in traditional use. The following hepatoprotective drugs are considered as standards which are already available in the pharmaceutical market. Since, our main objective is focused on the gene expression profile of Bax, Bcl2 and their role on
mitochondrial damage, it is very important to study the effect of different hepatoprotective drugs before and after toxicant treatment. Hence we have selected the following hepatoprotective drugs to be included in the objective.

2.3.1 Silymarin

Silymarin isolated from the seeds of *Silybum marianum* (Asteraceae) is a mixture of flavanolignans - silybin, silidianin and silychristin (Morazzoni and Bombardelli, 1995). The extract also contains other flavonoids, mainly taxifolin and quercetin (Gazak et al, 2007). All these compounds account for 65–80% of the whole extract content, with the remaining fraction being a chemically not well-defined fraction, composed mostly of polymeric and oxidized polyphenolic compounds (Willard, 1992). The mechanisms by which silymarin exerts its hepatoprotective action are under intensive investigation, and appear to be multifactorial. Silymarin and silybin prevent lipid-peroxide formation in liver cells, mainly due to their free-radical-scavenger properties (Pascual et al., 1993). Silymarin also has antifibrinogenic properties (Boigk et al., 1997), and is able to increase the synthesis rate of rRNA by activating RNA polymerase I (Machicao and Sonnenbichler, 1977); this enhances the biosynthetic apparatus, thus increasing the synthesis rate of both structural and functional proteins. Other than its antioxidant action, silymarin also reduces the turnover of membrane phospholipids and stabilizes the cell membranes of hepatocytes. Silymarin is prescribed under category as liver protectant and used in the treatment of hepatic disorders. Few marked brands of silymarin are Silybon (Micro labs), Silimar (Zy.Cad) and Sivylar (RanBaxy).

2.3.2 Lecithin

Lecithin, an important phospholipid is found in the major organs in our body such as the heart, the liver, and the kidneys (Iwata et al., 1993; Jimenez et al., 1990). Lecithin, a component of most cells, will help in transport and responsible for overall health of the body. Though it is produced within our own bodies, we do not always consume enough of the nutrition needed to produce it in adequate amounts (Iwata et al., 1993; Jimenez et al., 1990).
Lecithin is composed of phosphoric acid, choline, fatty acids, glycerol, glycolipids, triglycerides and phospholipids (e.g., phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol) (Iwata et al., 1993; Jimenez et al., 1990). However, lecithin is sometimes used as a synonym for pure phosphatidylcholine a phospholipid that is the major component of its phosphatide fraction. It may be isolated either from egg yolk or soya beans. Role of lecithin as hepatoprotective drug have been well studied and reported (Das and Vasudevan, 2006; Raj et al., 2010a). Lecithin is used in combination with silymarin (1:1 molar ratio) marketed as silipide (Morazzoni et al., 1992). The efficacy of silipide in cases of chronic hepatitis patients was found superior to silymarin (silybin) due to higher bioavailability (Buzzelli et al., 1993).

2.3.3 Catechin

Fresh tea leaves are rich in flavanol monomers known as catechins (Graham, 1992). Flavonoids have been found to play a very important role in protection against oxidative stress (Babich et al., 2005; Okada et al., 2001). Flavonoids are group of polyphenolic compounds that occur widely in fruit, vegetables, tea, cocoas and red wine (Arts et al., 1999; Matito et al., 2003). Studies have been carried out exploring different biological activities of catechins. Scavenging and productive activities of green tea polyphenols (Sato et al., 2010), preventive effect of green tea catechins on experimental tumor metastasis (Shimizu et al., 2010), protective effect of green tea on ethanol induced lipid peroxidation (Ostrowska et al., 2004) and tamoxifen-induced liver injury (Hesham et al., 2005) have been reported. There have been reports supporting the hepatoprotective effects of green tea against ethanol intoxication (Arteel et al., 2002; Baltaziak et al., 2004; Skrzydlewska et al., 2002). Effect of green tea and epigallocatechin gallate on ethanol-induced toxicity in HepG2 Cells was also reported (Sang et al., 2008). Proposed mechanism of catechin is its ability as powerful antioxidant and its free radical scavenging property.

2.3.4 L-Ornithin and L-Aspartate (LOLA)

L-Aspartate is an amino acid found in all forms of life. L-Aspartate is a dicarboxyl amino acid found in small amounts in body fluids. The body can synthesize l-aspartate, which makes it a non essential amino acid. L-Aspartate serves as a precursor for synthesis of proteins, oligopeptides, purines, pyrimidines, nucleic acids and L-arginine. L-aspartate is important for the proper
functioning of the body. Supplementation may be necessary if the body, due to physical strains or conditions, is unable to produce the required amount.

Ornithine, an amino acid, is manufactured by the body when another amino acid, arginine, is metabolized during the production of urea. Since ornithine is produced by the body, a deficiency of this nonessential amino acid can occur only during adverse conditions like severe trauma or malnutrition or some time during pregnancy (Zieve, 1986).

L Ornithin and L aspartate is available with trade name such as Hepacor (Intas), Lornit infusion (Zuventus) and Hepalon (Micro Nova). Ornithine aspartate has been shown to be beneficial in people with hepatic encephalopathy and liver cirrhosis. In a double-blind trial, people with cirrhosis and hepatic encephalopathy received either L-ornithine-L-aspartate or a placebo for two weeks (Stauch et al., 1998). Those taking the ornithine had significant improvements in liver function and blood tests compared with those taking the placebo.

It is widely believed that ammonia plays an important role in the multifactorial pathogenesis of hepatic encephalopathy (Butterworth et al., 1987; Ferenci et al., 1992; Mousseau and Butterworth, 1994). For all these reasons, reducing hyperammonia in patients with severe liver failure has always been one of the important goals of therapeutic applications. Administration of omithine and also ornithine compounds has been proved to decrease blood ammonia concentrations (Greenstein et al., 1956; Zieve et al., 1989; Nagao et al., 1989). During the last decades, clinical studies have shown that L-ornithine- L-aspartate reduces blood ammonia concentration, restores amino acid imbalances and may improve the clinical symptoms of hepatic encephalopathy in patients with mild liver failure (Staedt et al., 1993; Kircheis et al., 1993).

2.4 Expression studies involving Bax, Bcl-2 and P53

Fu et al., studied altered *in vivo* and *in vitro* expression of P53, Bcl-2 and Bax induced by microcystin-LR (MC-LR). Microcystins are a family of monocyclic heptapeptides produced by *Microcystis aeruginosa*. MC-LR is a hepatotoxin with high acute toxicity and potent tumor promoting activity. MC-LR is a potent inhibitor of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A), leading to increased protein phosphorylation which is directly related to its hepatotoxicity and tumor promotion activity. Therefore, by inhibiting PP1 and PP2A, MC-LR can target variety of key control proteins that regulate apoptosis. They studied the effect of MC-
LR on the rat hepatocyte BRL-3A. In this study they showed that the administration of MC-LR in vivo and in vitro can both significantly increase the Bax expression level. The level of Bcl-2 expression is significantly decreased in vitro and Bax/Bcl-2 ratio is increased more than two-folds and results were obtained in time and dose-dependent manner under certain conditions. But there was no significant change observed in Bcl-2 expression in vivo. First, this is attributed to the signaling pathways that regulate the status of phosphorylation of Bcl-2, which are more complex in vivo than in vitro. Second, this is due to the different mechanistic details that participate in the pure cell line and the in vivo toxicology machinery and partly due to the inter-individual variability, which is commonly observed in vivo.

Exley et al., studied expression of caspase and BCL-2 apoptotic family members in mouse preimplantation embryos. In this study they investigated which genes contribute to apoptosis in preimplantation embryos. Recent studies in mice and humans showed that the blastomeres of preimplantation embryos are susceptible to apoptosis. A few apoptotic cells can be observed in normal blastocysts also, which appears to be part of a normal developmental program. Additionally, it has been demonstrated that unfertilized eggs and polar bodies are eliminated via apoptosis. The signals that trigger apoptosis in embryos or the precise mechanism by which the apoptotic program is executed and controlled is not completely known. Because the preimplantation embryo develops in a unique, maternal environment, it is of great interest to determine whether the mechanism of cell death utilized by preimplantation embryos is similar to that of other tissues or whether it is highly specialized. In this experiment, oocytes were collected from superovulated female C57BL/6 mice. For the collection of embryos superovulated females were mated with single males. Preimplantation embryos were collected at 1-cell, 2-cell, 8-cell and blastocyst-stage. Expression of caspase and BCL-2 family genes in oocytes and preimplantation embryos were carried out using PCR amplification. Assay for Caspase Enzymatic Activity, BAX and BCL-2 were carried out using immunofluoroscence.

Lee et al., studied both apoptotic pathways regulated by Bcl-2 family in sodium fluoride-induced apoptosis of the human gingival fibroblasts (HGF). Fluorides exert a variety of effects in different cell types. In bone cells, fluorides elicit potentially beneficial effects by stimulating growth of bone cells. But there are rare adverse effects reported after long-term low dose fluoride ingestion. An overdose can cause serious acute toxicity. Fluoride has been found to inhibit
protein synthesis and cell cycle progression, as well as induce apoptosis in epithelial lung cells and alveolar macrophages. Cell viability assay, TUNNEL assay was carried out for the detection of apoptosis, detection of ROS species and caspase activity and western blotting for cytochrome c, Bax and Bcl-2. The cytotoxic effect of NaF on HGF was determined at various NaF concentrations and times and it was found that NaF inhibited the cell viability in a dose- and time-dependent manner. In TUNEL assay, the HGF showed apoptotic morphological changes including chromatin condensation, DNA fragmentation, which shows that fluoride induces apoptosis in HGF. The production of ROS was monitored using a fluorescence spectrometer with 2’, 7’-dichlorofluorescein diacetate (DCF-DA). It showed that NaF did not affect the ROS level in HGF. Caspase activity assay showed that NaF activates caspase-3 and caspase -9, resulting in apoptosis in HGF. In this study, Bcl-2 was shown to be downregulated and whereas Bax was not affected in NaF treated HGF. NaF resulted in an increment of cytochrome c release from the mitochondria into the cytosol in a dose-dependent manner. These findings suggest that NaF-induced apoptosis may be mediated mitochondria dependent pathways mediated by the Bcl-2 family with involvement of caspases cascade.

Siu et al., studied diclofenac-induced hepatocyte injury through Bax-mediated mitochondrial outer membrane permeabilization (MOMP) and protective roles of cyclosporin A (CsA). Diclofenac is a used as a nonsteroidal anti-inflammatory drug that is considered to be a safe and well tolerated medication. However, diclofenac has been reported to cause significant adverse effects, mainly mild hepatic injury and in rare but severe case, idiosyncratic hepatotoxicity. Bax acts a key regulator in inducing the mitochondrial outer membrane permeabilization (MOMP). When activated, Bax translocates to mitochondria, monomers of Bax and Bak can oligomerize to form large channels and induce formation of pores. These channels can allow apoptogenic factors and proteins sequestered in the mitochondrial intermembrane space to be released into the cytosol, resulting in apoptosis via activation of caspases and apoptosomes. In this study they hypothesized that the Bax/Bak pathway could initiate MOMP. Effects of diclofenac were studied using human HC-04 cells. To check weather diclofenac induces Bax activation and translocate to mitochondria, western blotting analysis was carried out which showed diclofenac did not alter the total cellular Bax expression levels in HC-04 cells but caused a time-dependent progressive increase in mitochondria-associated Bax. To explore the causal role and contribution of Bax
activation to diclofenac-induced hepatocyte injury, chemical inhibitor of Bax, CsA was used to
determine whether Bax inhibitors can rescue cells from injury. CsA fully prevented Bax and also
directly inhibited more upstream mediators activation by diclofenac. Apart from Bax inhibitor,
gene silencing using Bax siRNA was performed which resulted in complete ablation of
immunoreactive Bax. Thus this model showed that Bax plays a key role in mediating diclofenac-
induced lethal cell injury in human hepatocytes and the activation of both Bax and Bak involve
MOMP in diclofenac-induced cytotoxicity.

Bai J and Meng Z studied sulfur dioxide induced expression of apoptosis-related genes in livers
from rats. Apoptosis requires de novo synthesis of mRNA and proteins to take place, although
there is an overall decrease in RNA transcription. Certain critical genes required for apoptosis to
proceed, include P53, Bax and bcl-2. SO$_2$ is a ubiquitous air pollutant that is present in low
concentration in the urban air and in higher concentrations in the working environment. SO$_2$
pollution is produced by combustion and processing of sulfur containing fossil-fuels. SO$_2$ and its
derivatives can cause DNA damage and oxidative damage in multiple organs from mice
including liver. The mRNA and protein levels of P53, Bax and bcl-2 were analyzed in livers
using a real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) assay and
immunohistochemistry method. SO$_2$ inhalation, at all, tested concentrations showed an increase
in P53 mRNA levels in liver from rats (1.30-fold at 28 mg/m$^3$, 3.43-fold at 56 mg/m$^3$), but at
low concentration (14 mg/m$^3$) the increase in P53 mRNA levels was not statistically significant.
The increases in mRNA levels of Bax were 1.63-fold and 2.17-fold at the concentrations of 28
and 56 mg/m$^3$ compared with control group, respectively. But only a marginal increase was
detected in livers at the concentration of 14 mg/m$^3$. In case of bcl-2, SO$_2$ inhalation at 28 and 56
mg/m$^3$ caused statistically significant decreases of bcl-2 mRNA levels (0.63-fold at 28 mg/m$^3$
and 0.45-fold at 56 mg/m$^3$) in a dose dependent manner, whereas bcl-2 mRNA levels were not
significantly decreased in livers from rats treated with lower SO$_2$ concentration (14 mg/m$^3$).
Immunohistochemical analysis showed increase in P53 immunoreactive (IR) hepatocytes and
Bax protein in the livers 1 week after SO$_2$ inhalation. Whereas, dramtactical reduction in bcl-2 IR
hepatocytes appeared 1 week after SO$_2$ inhalation. Immunohistochemical staining also showed
that protein expression changes of P53, Bax and bcl-2 were predominantly observed in the portal
area in liver, and the staining was weak in central vein in livers from rats of all groups. Thus
results obtained in these studies indicated that SO\textsubscript{2} exposure can increase expression of P53 and Bax, and decrease the expression of bcl-2 in the liver of rats and the results might be associated to alterations in the oxidative stress, cytokines, and/or DNA damage.

Li et al., studied the roles of Akt and MAPK family members in silymarin’s protection against UV-induced A375-S2 cell apoptosis. Akt, the members of MAPK family, was found to be intimately involved in the inhibitory mechanism of silymarin on UV induced human malignant melanoma (A375-S2 cell) apoptosis. Akt protects cells from apoptosis by various stimuli, such as UV irradiation, ionizing irradiation, and different classes of chemotherapeutic drugs and DNA damaging agents. In this study cell viability was measured using MTT assay Expression studies were performed using western blotting analysis for Akt, P53, pro-caspase-3, Bax, bcl-2 phosphorylated Akt and P53. In this study, it was found that the expression of phosphorylated Akt was increased and P53 phosphorylation was reduced in a time-dependent manner by silymarin in A375-S2 cells. Proapoptotic mitochondrial protein, Bax, which caused the release of cytochrome c and the activation of post mitochondrial caspase cascade, gets activated by P53. Silymarin attenuated the expression of Bax slightly, but increased the expressions of Bcl-2 in UV-irradiated A375-S2 cells. These results indicated that silymarin prevents A375-S2 cells from UV-induced apoptosis by down-regulation of phosphorylated P53 levels after activation of Akt, followed by decreased expression of Bax and increased expressions of Bcl-2.

In UV carcinogenesis, silibinin has been shown to up-regulate the expression of P53 protein in mouse skin. Inhibition of skin carcinogenesis by silymarin has been primarily associated with its effect at the tumor promotion stage. However, the molecular mechanisms associated with the chemopreventive effects of silymarin are not clearly understood. Katiyar et al studied silymarin induced apoptosis to determine whether P53, the proteins of Bcl-2 family and caspases were involved in the process. To accomplish this, the effect of silymarin was determined on the preneoplastic epidermal cell line JB6 C141 which is a well-developed and excellent cell culture model for studying tumor promotion. The results of this study show that silymarin induces apoptosis primarily in a P53-dependent pathway in JB6 C141 cells, which was further confirmed by using P53 wild-type (P53\textsuperscript{+/+}) and P53-deficient (P53\textsuperscript{-/-}) fibroblasts. The function of activated P53 is critically dependent on the phosphorylation of P53 protein at the Ser\textsuperscript{15} residue. To
determine the possible role of P53 in induction of apoptosis by silymarin in JB6 C141 cells, the expression of total P53, and phosphorylated P53 was accessed by Western blot analysis. The treatment of silymarin resulted in a dose dependent increase in the basal level of P53 protein as well as increase in its phosphorylation at Ser\textsuperscript{15}. To check whether tumor suppressor protein P53 is required for induction of apoptosis caused by silymarin, the apoptotic effect of silymarin were determined also on fibroblast cell lines derived from P53-deficient (P53\textsuperscript{-/-}) mouse embryos. Result from these studies indicated that P53-deficient cells are less susceptible to silymarin-induced apoptosis, which supported the notion that silymarin-induced apoptosis in preneoplastic epidermal JB6 C141 cells may be mediated primarily through the involvement of tumor suppressor protein P53. Treatment of JB6 C141 cells with silymarin resulted in a dose dependent decrease in the levels of proapoptotic Bcl-2 and Bcl-xl proteins expression and upregulation of proapoptotic Bax protein. But sylamarin had little effect on Bcl-2 and Bax expression in the P53-deficient cells. Thus the results of this study indicated that silymarin-induced apoptosis in JB6 C141 cells primarily mediated through a P53-dependent pathway, and P53-independent pathway seems to have a minor role.

2.5 Studies related to mitochondrial damage and cell death

Mitochondria are present in almost all eukaryotic species. Mitochondria are shielded from the cytoplasm by an inner and an outer membrane. Mitochondria provide energy in the form of ATP by a process called oxidative phosphorylation. Cells obtain ATP, which are produced at the matrix side of the inner mitochondrial membrane. Besides being guardians of survival, mitochondria also harbor noxious molecules in the intermembrane space. Most proapoptotic signaling pathways, including those induced by DNA damage, growth factor depletion and cytosolic calcium overload, were found to converge on the mitochondria. A wide variety of apoptotic signals activate proapoptotic Bcl-2 members such as Bax, Bak and Bid, resulting in a disturbed balance between pro- and antiapoptotic Bcl-2 family proteins. As a consequence, outer mitochondrial membrane integrity is lost due to the oligomerization of proapoptotic Bcl-2 members in the mitochondrial membrane and the formation of a megapore complex. This results in the permeabilization of the membrane and the release of proteins from the inter membrane space. Cytochrome c is one of the protein released from mitochondria under apoptotic
conditions. Cytochrome c sparks a caspase cascade by promoting apoptosome formation provided that apoptotic protease-activating factor-1 (Apaf-1) is expressed.

Alcohol abuse is the principal factor for the cause of serious liver diseases. The accurate mechanism of ethanol-induced hepatic cells injury is still not completely clear. Some researchers found that ethanol could activate lipid peroxidation, leading to liver injury. Mitochondrion is one of the organelle where reactive oxygen species (ROS) are formed. Yan et al studied the role of mitochondria in alcoholic liver disease (ALD) and ethanol induced mitochondria injury. In this experiment rats were administered with different concentration of ethanol and liver samples were collected. Liver samples were analyzed for mitochondrial PTP opening and intracellular calcium concentration. It was observed that the mitochondria were damaged, and membrane and cristae were broken or disappeared under electron microscopy. This experiment also indicated that long-term alcohol abuse of ethanol can make PTP open. The mechanism for the opening of PTP is possibly because in hepatocytes, anti-oxidation materials are reduced, and or lipid peroxidation intensifies, causing injury to the mitochondrial membrane. Thus, because of increase in membrane permeability, mitochondria swell, and finally injured. Physiologically, hepatocytes are surrounded with a high concentration of Ca\(^{2+}\). Distribution of Ca\(^{2+}\) is uneven intracellularly, the content of Ca\(^{2+}\) is higher in mitochondria and endoplasmic reticulum than in cytoplasm. Slow accumulation of Ca\(^{2+}\) in mitochondria causes overloading of Ca\(^{2+}\) so as to make PTP open, leading to a prompt decrease of the membrane potential, swelling of mitochondria, and finally apoptosis. In this study it was found that the Ca\(^{2+}\) level of the ethanol administered group was significantly higher than that of the control group. It indicates that Ca\(^{2+}\) plays an important role in the process of ALD. The Ca\(^{2+}\) signal may induce mitochondrial damage through several possible ways as follows: (1) the elevation of Ca\(^{2+}\) density in hepatocytes makes PTP open, causing Ca\(^{2+}\) to transfer from cytoplasm to mitochondria. This damage is reversible; (2) the overloading of Ca\(^{2+}\) in mitochondria maintains PTP open, leading to a decrease of membrane potential, swelling of mitochondria, and finally apoptosis. So this study indicated that the Ca\(^{2+}\) signal transduction system plays a crucial role in the pathogenesis of ALD. Reports from these studies indicated that mitochondrial damage must be of pathophysiological importance in the process of ALD. The pathway is possibly Ca\(^{2+}\) transferring from cytoplasm to mitochondria,
increasing mitochondrial permeability, decreasing mitochondrial membrane potential, finally leading to hepatocyte injury.

Mammalian cell survival is largely dependent upon extracellular signals transduced by Akt. Akt is also known as protein kinase B (PKB), which is capable of inhibiting apoptosis induced by a variety of proapoptotic stimuli. Akt inhibits mitochondrial cytochrome c release, a critical early event in the mammalian apoptotic cascade. The precise mechanism whereby Akt inhibits this release is not completely clear. Changes in outer mitochondrial membrane (OMM) permeability permit cytochrome c translocation from the mitochondrial intermembrane space (IMS) to the cytosol, where it contributes to apoptosome formation in response to proapoptotic stimuli. A dynamic multiprotein complex brings the OMM and IMM (inner mitochondrial membrane) into close proximity to each other, forming mitochondrial contact sites. The OMM voltage-dependent anion channel (VDAC) and the IMM adenine nucleotide translocator (ANT) are integral components of this multiprotein complex. Sustained closure of VDAC could lead to mitochondrial dysfunction, matrix swelling, and OMM rupture by limiting mitochondrial ADP availability. The glycolytic enzymes hexokinase I and II (HKI and HKII) bind mitochondria with high affinity at OMM contact sites where they interact with VDAC. The association between mitochondrial HK (mtHK) and the OMM is dynamic and greatly influences the structure and conductance of VDAC, which may have regulatory implications for cytochrome c release. In addition to regulating VDAC activity, mtHK may directly antagonize the recruitment of Bax to the mitochondria. Expression of an activated form of Akt increases basal levels of mtHKs at the mitochondria and that overexpression of HKI and a functional variant of HKII can attenuate apoptosis. Majewski et al studied Akt mediated hexokinase-mitochondria interaction influencing apoptosis. Activated Akt increases association of hexokinase with mitochondria and inhibits this cytochrome c release and apoptosis in Bax- and Bak-deficient cells. Thus, the permeability of the OMM and apoptosis can occur in the absence of Bax and Bak, which are inhibited by Akt through maintaining the association of mtHKs with mitochondria. In contrast, activated Akt is insufficient to inhibit apoptosis induced by Ca$^{2+}$ release whether or not Bax and Bak are present. Reports obtained from these studies showed that growth factors and Akt can maintain mitochondrial integrity by increasing HK association with mitochondria. In the absence of signals to maintain Akt-dependent HK association with mitochondria, some apoptotic stimuli,
such as UV irradiation, remain capable of eliciting cytochrome c release and apoptosis in the absence of Bax and Bak, which is not suppressed by Bcl-2. Such a mechanism may contribute to observed apoptotic cell death in organisms lacking Bcl-2 proteins. Though complete mechanism for the dissociation of HK from mitochondria to induce apoptosis is not known, results obtained from these studies showed that mtHKs play a critical role in mammalian mitochondrial-dependent apoptosis.

Ligeret et al studied protective effects of silibinin on mitochondria in cold preservation–warm reperfusion liver injury. The most biologically active component of silymarin is silibinin, a polyphenolic molecule, also used as hepatoprotectant. Initial graft nonfunction in liver transplantation is caused by the succession of the two phases necessary for transplantation: the cold preservation and the warm reperfusion. During cold preservation, ischemia induces a substantial decrease of mitochondrial respiratory chain activity, subsequent disturbances in membrane ion translocation and cytoskeletal disruption resulting in eventually irreversible membrane damages. The reperfusion phase can amplify ischemic injury. First, reperfusion promotes the generation of reactive oxygen species (ROS) that cause nonspecific oxidative damage to lipids, proteins and DNA. Second, reperfusion induces mitochondrial permeability transition causing swelling, membrane potential decrease and ultimately total mitochondria dysfunction leading to necrotic and apoptotic cell deaths. Mitochondria were isolated from liver and mitochondrial respiration, lipid peroxidation, superoxide anion and mitochondrial ATP was measured. The presence of silibinin in the preservation solution markedly decreased lipid peroxidation induced by preservation and reperfusion, O$_2^{•−}$ release and induced a significant rise of ATP. These results indicated that silymarin increased ATP level and reduced O$_2^{•−}$ generation levels. Therefore, these studies suggest that silibinin could be responsible, at least in part, to the beneficial effect of silymarin and it has strong potential to counteract damages induced to the liver by cold preservation and warm reperfusion. Silibinin exerted this beneficial action notably by completely suppressing oxidative stress and by improving mitochondrial functions.