1. Introduction

1.1 The liver

The liver is one of the most important vital organs in the human body. Liver plays a significant role not only in metabolism and detoxification of exogenous toxins and therapeutic agents, but also in the bioregulation of fats, carbohydrates, amino acids, proteins, blood coagulation and immunomodulation.

Many toxins target the liver and cause hepatotoxic effects that can be observed through some biochemical parameters. Impairment of the liver generally occurs from excessive exposure to xenobiotics, alcohol, chemotherapeutic agents, virus and protozoan infections. Depending upon the severity of toxicant insult, hepatic cell injury can lead from acute to chronic hepatitis, which if left untreated can result in cirrhosis or malignant lesions.

1.2 Liver toxicity

The great susceptibility of the liver to damage appears to be a consequence of its primary role in the metabolism and disposition of foreign substances. The concentration of foreign chemicals in the liver, the metabolic conversions that occur in the hepatocytes, the position of the liver as a portal to the tissues, and the excretion of the agents or their metabolites in the bile all contribute to the special vulnerability of the liver to chemical injury.

1.3 Mechanisms of chemical-induced liver injury

Different mechanisms of chemical-induced liver injury can be divided into following types

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Table taken from (Lee, 2003)
1.3.1 Disruption of calcium homeostasis and cell membrane injury

The ability of a cell to maintain the required amount of intracellular calcium concentration is called as calcium homeostasis. Generally cytosolic free calcium is maintained at a very low concentration compared to the extracellular levels in the healthy cell. The balance of intracellular ions is maintained by a variety of energy consuming processes including the $\text{Ca}^{2+}$ and $\text{Mg}^{2+}$ ATPases. Bioactivation of certain drugs by the cytochrome p450 system can engender reactive intermediates that can bind to various cellular proteins leading to widespread cellular dysfunction. Drug-induced damage to cellular proteins that are involved in ion balance can lead to an influx of calcium that disrupts, among other processes, normal actin filament assembly and disturbs ATP production. The resulting dispersal of the cytoskeleton leads to blebbing of the cell membranes and, if cell membrane disruption is of sufficient magnitude, irreversible cell injury and cell lysis can occur.

1.3.2 Canalicular and cholestatic injury

Cholestasis is a condition where bile cannot flow from the liver to the duodenum. This condition is marked by the slow progressive destruction of the small bile ducts (bile canaliculi) within the liver. When these ducts are damaged, bile builds up in the liver (cholestasis) and over time damages the tissue. This can lead to scarring, fibrosis and cirrhosis. More than 30 drugs have been identified that can lead to cholestasis (Lewis, 2000). Some drugs bind to canalicular transporter molecules and lead to the arrest of bile formation or movement within the lumen of the canalicular system (Trauner et al., 1998). Another mechanism leading to cholestasis involves disruption of actin filaments situated around the bile canaliculi preventing the normal pulsatile contractions that move bile through the canalicular system to the bile ducts. Drugs that bind to actin filaments such as phalloidin and those that affect calcium homeostasis and cellular energy production can also generate this type of injury.

1.3.3 Metabolic bioactivation by cytochrome p450 enzymes

The cytochrome P450 superfamily (officially abbreviated as CYP) is a large and diverse group of enzymes. CYP P450 enzymes are mainly present in the inner membrane of mitochondria or in the endoplasmic reticulum of liver cells. These CYP P450 enzymes metabolize endogenous and exogenous toxins, drugs, xenobiotics and other potentially harmful molecules. This
biotransformation is a 2 step process; termed phase 1 chemicals are bioactivated to a high energy reactive intermediate molecule, in preparation for the second step, phase 2, which involves formation of covalent bonds with polar molecules such as glucuronic acid. Conjugation forms a water-soluble metabolite that can be excreted. However, in some circumstances, such as overdosage, the high-energy reactive metabolites can form adducts that are covalent bonds with other cellular constituents such as proteins and nucleic acids. In acute toxicity, adducts can form with essential cellular enzymes leading to cell injury or death. The site of toxic cellular injury within the hepatic acinus reflects the site of bioactivation of the chemical. For instance, carbon tetrachloride is metabolized by the cytochrome P450 system to CCl$_3^\cdot$, a free radical that induces cell membrane injury. Lesions induced by carbon tetrachloride are most severe in the periacinar (centrilobular) areas, because this is the area where the smooth endoplasmic reticulum is most abundant, and, therefore, where the active form of the chemical is present in greatest concentration. Consequently, the centrilobular region of the hepatic lobule is by far the most common site of acute toxic injury. Acetaminophen toxicity is another and more commonly encountered example of this mechanism of liver injury (Zhang et al., 2002).

1.3.4 Stimulation of autoimmunity

In addition to direct damage to cellular proteins and nucleic acids, adduct formation can lead to immune mediated liver injury. This can occur when adducts form between drug metabolites and cellular proteins or nucleic acids and generate neoantigens. These neoantigens may be formed on the cell surface by interactions of chemicals with certain cell membrane receptors or they may be processed, transported to the cell surface and presented as antigens. This process has been demonstrated with several drugs that form adducts with the cytochrome P450 isoforms that are involved in their metabolism, including tienilic acid and dihydralazine. Depending on the MHC context of antigen presentation both cellular and humoral immunity can be involved (Lewis, 2000). Injury can occur through direct cellular cytotoxicity and antibody dependant cellular cytotoxicity. Hepatic injury may be significantly exacerbated by recruitment of inflammatory cells such as neutrophils and activation of sinusoidal lining cells, particularly Kupffer cells (Jaeschke, 2000).
1.3.5 Stimulation of apoptosis

Apoptosis is a form of cell death characterized by organized nuclear and cellular fragmentation (Rust and Gores, 2000). In the course of apoptotic death intact cell organelles and cell membranes are fragmented into small membrane bound bodies. Cellular DNA is cleaved by endonucleases to 120–180 base pair fragments. Classically apoptosis can be triggered through two basic mechanisms in the hepatocyte; interactions between death ligands (Fas-ligand, TNF) and death receptors (Fas and TNFR-1) that trigger caspase 8 activation or damage to mitochondrial inner membranes releasing cytochrome c that binds to Apaf-1 activating it, leading to downstream activation of caspase 9. These pathways are not completely separate as a protein named Bid that is activated by caspase 8 can cause mitochondria to release cytochrome c. Certain chemicals may be able to trigger apoptosis by direct stimulation of pro-apoptotic pathways in the hepatocytes. Alternatively, apoptosis can be stimulated by several other pathways including immune-mediated events that lead to the release of tumor necrosis factor alpha or activate Fas pathways. Chemicals that damage mitochondria can also stimulate apoptosis through the release of cytochrome c. Cholestasis can also stimulate apoptosis through the action of pro-apoptotic bile acids such as glycodeoxycholic acid (GCDC) (Bissel et al., 2001). This type of toxicant-induced transport of death receptors represents a novel form of hepatocyte injury. Additional pathways may involve triggering of apoptosis by protein kinase C activation and mitochondrial injury.

1.3.6 Mitochondrial Injury

Chemicals that damage mitochondrial structure, enzymes or DNA synthesis can disrupt β-oxidation of lipids and oxidative energy production within the hepatocytes (Bissel et al., 2001). Prolonged interruption of β-oxidation leads to microvesicular steatosis within hepatocytes. Mild insult leads to macrovesicular steatosis. In severe cases microvesicular steatosis, hepatic failure and death can result. Some drugs may inhibit β-oxidation (asprin, valproic acid, tetracyclines) and others may disrupt oxidative phosphorylation alone or in addition (bile acids, amiodarone) depleting the hepatocytes of energy. Certain antiviral dideoxynucleoside analogues can disrupt mitochondrial DNA synthesis through inhibition of DNA polymerase gamma, leading to depletion of mitochondrial DNA and leading to hepatocyte death.
With respect to the above methods of liver damage, canalicular injury, cholestatic injury, disruption of calcium homeostasis, bioactivation of chemicals via cytochrome P450, fatty liver and fibrosis have been extensively studied and reported. Newer understanding of injury at the molecular level has given a focused approach; hence we concentrated our studies on apoptosis and mitochondrial damage.

1.4 General features of apoptosis

Until the 1970’s necrosis was the only one form of cell death (Haustetter & Izumo 1998). In 1972 Kerr and colleagues identified an alternative mechanism of cell death which was named apoptosis based on the Greek word for “falling off” (Kerr et al., 1972). Necrosis occurs when a cell dies by “accident” (Haustetter & Izumo 1998) as the result of a powerful insult such as ischemia, bacterial or viral infection, or lack of ATP production. Cells undergoing necrosis swell resulting in a loss of membrane integrity and the release of their cytosolic contents. Leakage of these internal contents causes neighboring cells to also die by necrosis and activate the immune system (Allen et al., 1997). Apoptosis, otherwise known as programmed cell death, is the result of cell suicide (Haustetter & Izumo 1998; Allen et al., 1997). The function of apoptosis is to remove unwanted cells, to counterbalance cell proliferation, and to help develop organ architecture. Cells undergoing apoptosis present distinct and unique morphological features when the process is almost complete, this is referred to as end-stage apoptosis. These characteristics include chromatin condensation resulting in the nucleus looking like a “half-moon”, as well the cell shrinks in size, and the membrane blebs (seen only in vitro) (Allen et al., 1997). Another hallmark of apoptosis is that DNA of the cell becomes cleaved at 180-200 base pairs intervals which appear as a “ladder” when electrophoresed on an agarose gel (Allen et al., 1997). Apoptotic cells do not activate the immune system; instead they are engulfed by phagocytic cells including macrophages/monocytes and by other nearby non-phagocytic cells such as vascular smooth muscle cells. It is believed that these phagocytic cells recognize a cell as being apoptotic if there is an increase in the number of phosphatidylserine residues expressed on the cell’s outer membrane (Allen et al., 1997). The entire apoptotic process occurs within hours (Colucci, 1997).
1.4.1 Regulators of apoptosis

1.4.1.A) Tumor suppressor protein P53

The P53 tumour suppressor participates in the initiation of the apoptosis cascade in response to DNA damage (Burns and El-Deiry, 1999). The human P53 protein acts as a transcription factor and plays a key role in the cells response to genotoxic stress such as DNA damaging agents, hypoxia, nucleotide depletion and oncogene activation. It performs many biochemical functions as well as regulates multiple cellular processes, including cell proliferation and DNA repair. Under normal conditions in most cells and tissues, the P53 is expressed at barely detectable low levels and has a rapid turnover rate with its half-life being on the order of 20-30 min (Wiman, 1997; Burns and El-Deiry, 1999). In response to the aforementioned stressors, the P53 protein level increases, and it accumulates through protein stabilization and initiates the response pathways. The increased DNA binding activity mediates these responses by regulating (either activating or inhibiting) transcription of target genes containing specific DNA binding sites for P53 domains. The P53 responsive genes include the p21, a nuclear factor that acts on cell cycle progression, pro-apoptotic proteins in the Bcl-2 family (e.g. Bax and Bcl-xS), and gadd (growth arrest and DNA damage repair factor) (Tang et al., 1998; Burns and El-Deiry, 1999). Although our present knowledge in this area remains unclear, available evidence has shown that there are both transactivation-dependent and –independent functions of P53 in triggering the apoptotic program (Bellamy, 1997). MDA-MB-231 breast cancer cells treated with the isoflavonoid, genistein (5-30 µmol/L, 24-72 h) inhibited cell growth and induced apoptosis through a P53-independent pathway (Li et al., 1999). The dependence of P53-activated apoptosis may also be animal or cell specific. Russo and Russo (2000) reported that rats treated with human chorinoic gonadotropin (hCG) showed a P53-dependent activation of apoptosis with partial dependence on the Bcl-2 family related genes (Bcl-xL). Human breast epithelial MCF-10F cells and urothelial T24 cells showed different responses to hCG treatment cells (Srivastava et al., 1998). The incubation of hCG (100 IU/mL, 24 h) reduced cell proliferation and elevated the expression of P53, Bax and p21 in MCF-10F cells, while no changes in gene expressions were observed in T24 human breast epithelial. A recent study found that single-stranded DNA triggered P53-dependent apoptosis in mammalian BALB/c 3T3 cells, and in response was significantly lower in P53 null cells (Nur-E-Kamal et al., 2003).
1.4.1.B) Bcl-2 Family

Members of the Bcl-2 family represents a major class of intracellular regulators or modulators in the apoptosis pathway. They are present in the endoplasmic reticulum, nucleus and outer mitochondrial membranes. Bcl-2 family includes both pro-apoptotic and anti-apoptotic proteins. The best characterized pro apoptotic Bcl-2 family member are Bax and Bid, while the well known anti-apoptotic members include Bcl-2 and Bcl-xL (Pellegrini and Strasser, 1999). The pro apoptotic members of the Bcl-2 family have also been implicated in regulating the release of mitochondrial cytochrome c, which in turn activates a subsequent caspase cascade (Nieves-Neira and Pommier, 1999; Waterhouse and Green, 1999).

1.4.1.C) Caspases

Caspases function as effectors, particularly in the execution phase of the cascade of events ultimately leading to cell death. They are proteolytic enzymes that have the amino acid cysteine (Cys) in their active sites and cleave their target proteins at specific aspartic acid (Asp) residues. Each consists of a large, inactive precursor (a procaspase), which is itself activated by cleavage at Asp by other Caspases. Therefore, prompted by adapter proteins transduced upon receipt of potentially apoptotic signals, Caspases are activated to initiate sequentially amplified proteolytic cascade to control apoptosis (Cohen, 1997; Janicke et al., 1998). These activations results in the cleavage of critical cellular substrates, including poly ADP ribose polymerase (PARP) and nuclear membrane, dismantling of the nucleus by DNAs as well as detachment of neighboring cells to facilitate phagocytic actions (Allen et al., 1997).

1.4.2 Mitochondrial involvement in apoptosis

Mitochondria are frequently involved in the apoptosis process (Green D, Kroemer G., 1998; Cai et al., 1998; Green & Reed., 1998; Susin et al., 1998). Mitochondria have the ability to release multiple proteins including cytochrome c, apoptosis inducing factor (AIF), caspase 2, procaspase 9 and Dnase. Release of cytochrome c and apoptosis inducing factor ensure activation of the apoptotic pathway. Cytochrome c normally functions within the mitochondria as an electron chaperon between complex III (ubiquinol) and complex IV (cytochrome oxidase), which is involved in electron transport chain (Cai et al., 1998). Release of cytochrome c into the cytosol occurs with or without permeability transition pore (PT) opening and with or without decreased
mitochondria transmembrane electrical potential (Susin et al., 1998). (Finucane et al., 1999). Once in the cytosol, cytochrome c can interact with dATP/ATP, pro-caspase 9 and apoptosis protease activating factor (APAF) -1 to activate caspase 9. AIF can also be released through the PT pore of the mitochondria causing volume dysregulation which can result in apoptosis (Green D, Kroemer G., 1998). AIF and cytochrome c can induce release of each other thereby providing an overlap in apoptotic signaling (Cai et al., 1998). The PT pore can be activated by Caspases, oxidants, calcium (>>10 µM), low ATP levels, reduced mitochondrial transmembrane electrical potential, nitric oxide and by Bax (Green D, Kroemer G., 1998; Cai et al., 1998; Green & Reed., 1998; Susin et al., 1998; Susin et al., 1999). The PT pore connects the inner and outer membranes of the mitochondria and participates in regulating matrix calcium, pH, mitochondrial transmembrane electrical potential and volume (Susin et al., 1998). Thus, mitochondria are also involved in regulating apoptosis.

**Figure 1.1** Mitochondrial pathway in triggering apoptosis. Pro-apoptotic Bcl-2 family members translocate from the cytosol to mitochondria by cellular stress. Cytochrome c is released, that catalyzes the oligomerization of Apaf-1 (apoptotic protease activating factor 1). Apaf-1 recruits and promotes the activation of pro-caspase-9. This, in turn, activates pro-caspase-3, leading to apoptosis (Zimmermann et al., 2001)
Many toxic drugs cause either apoptosis or necrosis, depending on their dose used. What determines, whether the injured cell undergoes apoptosis or necrosis? There are suggestions, that the availability of ATP is critical in determining apoptosis, whereas the depletion of ATP leads to necrotic cell death (Lelli, 1998). Toxic substances may induce apoptosis at low concentrations or early after exposure whereas necrosis can occur later at higher concentrations. The induction of both forms of cell death by cytotoxic agents may involve similar metabolic disturbances and above all, mitochondrial permeability transition (mPT) (Kroemer et al., 1995). Whereas blockers of mPT (e.g. cyclosporine) prevent both apoptosis and necrosis. Whether apoptosis or necrosis occurs, depends on the number of mitochondria affected, as illustrated in Figure 1.2.

**Figure 1.2** Cell death regulation by mitochondria. The decisive factor in triggering apoptosis or necrosis or in cell survival is the number of mitochondria involved.

Hence in this present work, we made an attempt to find out if the liver toxicants such as CCl-4, D-Galactosamine, alcohol, drugs like paracetamol, anti TB drugs (isoniazid: Rifampicin: pyrazinamide) would causes any mitochondrial damage leading to cell death. Further to find out if the liver toxicants could bring the changes in the levels of P53, Bax and Bcl-2. Because over/under expression of these genes will have a direct effect on the mitochondrial membrane potential and subsequently release of cytochrome c finally leading to apoptosis. We also made an attempt to study the effect of selected hepatoprotective drugs (catechin, lecithin, L-ornithin-L-aspartate, L-ornithin and silymarin) on mitochondrial damage if any caused by the toxicants.