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MOLECULAR MECHANISMS OF LIVER INJURY INDUCED BY HEPATOTOXINS

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ABSTRACT
Liver is a vital organ and plays a major role in detoxification and excretion of many toxic chemicals (hepatotoxins). To detoxify the hepatotoxins liver cells operate various metabolic pathways involving different enzyme systems. During this process different kinds of toxic free radicals and electrophiles are produced and these molecules are responsible for the liver injury that leads to hepatic dysfunction. The pathways and the molecular mechanisms involved during the pathogenesis of liver injury due to abuse of paracetamol (acetaminophen), alcohol, carbon tetrachloride (CCl_4), and N-nitrosodiethylamine (NDEA or DEN) and N-nitrosodimethylamine (NDMA) are reviewed in this article to understand their adverse cellular reactions which are useful in devising new modalities for the treatment of liver injury induced by hepatotoxins. Metabolism of paracetamol involves glucuronidation (UDP-glucuronosyl transferases), sulfation (sulfotransferases) and deacetylation (N-deacetylase) pathways. At higher doses, cytochrome P450 (CYP2E1) enzyme system is involved and a toxic metabolite namely N-acetyl-p-benzoquinone imine (NAPQI) is produced. In alcohol metabolism, alcohol dehydrogenase and aldehyde dehydrogenase enzyme systems are implicated and acetaldehyde, a highly toxic molecule is formed during the reaction. When alcohol is consumed at large quantity CYP2E1 enzyme system is activated and resulted in the production of ROS such as superoxide (O2• −), hydroxyl radical (OH.) and hydrogen peroxide (H_2O_2) and other free radicals. CCl_4 metabolism is catalyzed by CYP2E1enzyme system and the reaction ends with the production of highly toxic free radicals such as trichloromethyl radical (CCl_3*) and trichloromethyl peroxy radical (CCl_3OO*). Metabolism of NDEA and NDMA involves alpha-hydroxylation pathway which produce hydroxymethylnitrosamine and it is further transformed into diazohydroxide and formaldehyde. The in vivo antioxidant molecules particularly reduced glutathione (GSH) are involved in the detoxification of free radicals. When detoxification process fails, the toxic intermediate metabolites can potentially alter the structure and function of cellular macromolecules, leading to apoptosis and/or necrosis and establish the liver injury.

KEYWORDS: Liver injury, hepatotoxins, molecular mechanisms, paracetamol, carbon tetrachloride, alcohol, N-nitrosoamines.

INTRODUCTION
Liver is an important organ which plays a pivotal role in regulating various physiological processes of the body. It has great capacity to detoxify toxic substances and synthesize useful molecules. It has unique capacity to regenerate its cells.[1,2] Because of its strategic location, liver receives all ingredients of blood circulation from the small and large intestines, as well as spleen and pancreas, through the portal vein. The hepatocytes are the unique functional cells of the liver and make up 70-85% of the liver's mass. They are functioning as a biochemical re-processor of absorbed food ingredients, which include metabolic, secretory and storing functions. Liver performs metabolism of lipids, carbohydrates and proteins and regulates levels of amino acids in the blood.[3] It is involved in the biosynthesis of biomolecules like glycogen from glucose by glycogenesis, lipoproteins from lipids, plasma proteins, several plasma carrier proteins, clotting factors and albumin and it is also a storage organ for glycogen, triglycerides, vitamins and excretion of urea from the blood stream. Further, liver is the largest secretory gland of the body, producing bile which is secreted into stomach through bile ducts. Bile is essential for digestion and absorption of fat and lipophilic nutrients. Furthermore, the liver is a part of the endocrine system which synthesizes five important hormones such as Insulin-like Growth Factor-1 (IGF-1), Angiotensinogen, Thrombopoietin, Hepcidin and Betatrophin. As a major regulator of plasma glucose and ammonia levels, liver is essential for optimal function of the brain. Loss of liver function leads to chronic hepatic encephalopathy and eventually coma.[4] Apart from all these important functions, liver provides first line defense against toxic

molecules entering in to it along with nutrients through portal blood. The vital role played by the liver is the detoxification and elimination of toxic chemicals exposes to it, otherwise they may cause injury to it. Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotics.[5] Hepatotoxins are exogenous compounds of clinical relevance and may be included overdoses of certain drugs, industrial chemicals, environmental pollutants and certain herbal dietary supplements (such as Chaparral and MaHuang) and toxins occur in natural products such as haloalkanes, *Amanita* poison, *Bacillus cereus* emetic toxin and cyanobacteria microcystins.[6] The adverse effects of hepatotoxins depend up on the chemical entity, point of entry and speed of assimilation and the health condition of intoxicated person.[7]

Details of metabolic alterations and molecular mechanisms of hepatic damage induced by hepatotoxins need to be thoroughly investigated, because therapeutic options for curing liver damage depend up on the correct understanding of molecular mechanisms of pathogenesis underlying in hepatotoxicity. Even though several hepatotoxins are reported, the discussion in the present article is restricted to commonly used drug such as paracetamol and the xenobiotics which are frequently exposed by the people such as alcohol, CCl_4 and nitrosamine compounds like NDEA/DEN) and NDMA.

PARACETAMOL

Paracetamol, a synthetic compound, also known as acetaminophen, is widely available as over-the-counter (OTC) drug and it is used for analgesic (pain relieving), anti-inflammatory and antipyretic actions.[8] Chemically paracetamol is a phenolic compound [N-acetyl-*p*-aminophenol (APAP remains inadequate. Involvement of serotogenic, opioid, nitric oxide (NO) and cannabinoid pathways, and/or a combination of interrelated pathways are reported in the mechanism of analgesic action of paracetamol.[9,10]

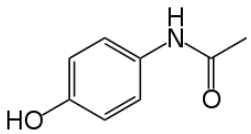

Fig. 1: Chemical structure of paracetamol.

Although it is safe at therapeutic doses, overdose of paracetamol can cause severe liver injury. Davidson and Eastham .[11] were the first to report that acetaminophen was hepatotoxic in overdose. At the therapeutic adult oral dose of 1-2 g/day, paracetamol is indicated for fever and mild to moderate acute pain conditions. Maximum recommended therapeutic dose of paracetamol is 4 g/day in adults and 50-75 mg/kg/day in children. Consumption of a single dose greater than 7 g in an adult and 150 mg/kg in a child is considered as potentially toxic to the liver and kidneys. The liver is the main target organ for acute paracetamol toxicity. Overdose of paracetamol is the leading worldwide cause of drug induced acute liver failure (ALF). Toxicity may occur even within the recommended dose range in certain patient groups because of their altered metabolism. There have been a number of reports on paracetamol toxicity in children after the introduction of the i.v. formulation. Several factors, such as concomitant alcohol abuse, concurrent medications, genetic factors and nutritional status can influence the susceptibility and severity of paracetamol induced hepatotoxicity.[12,13]

Molecular mechanism of liver damage induced by paracetamol

The liver, and to a lesser extent the kidneys and intestine, are the major organs implicated in the metabolism of paracetamol. It is metabolized via several metabolic pathways including glucuronidation (glucuronosylation), sulfation and deacetylation. After a therapeutic dose, paracetamol is mostly converted to pharmacologically inactive glucuronide (addition of glucuronic acid to paracetamol). (paracetamol-glucuronide, 52-57%) and sulfation (paracetamol-sulfate, 30-44%) These two conjugates are relatively non-toxic metabolites, which are excreted out of the body as urinary metabolites. [14]

Glucuronidation pathway of paracetamol metabolism is catalyzed by UDP-glucuronosyl transferases (UGTs). UGTs modify the paracetamol molecule to more water-soluble by transferring the glucuronosyl group from UDP-glucuronic acid. Studies in human liver microsomes and cultured hepatocytes indicate that isozymes such as UGT1A1, UGT1A6, UGT1A9 and UGT2B15 are involved in paracetamol glucuronidation. A family of cytosolic enzymes, called sulfotransferases (SULTs) carries out sulfation of paracetamol. SULTs transfer a sulfo group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to paracetamol, making it more polar and prone to elimination. From the liver, most of glucuronide and sulfate conjugates get transported into the kidneys through the blood stream and excreted.[15] At over doses of paracetamol (more than 4 g/day), glucuronidation and sulfation pathways become saturated, cytochrome P450 enzymes (CYP) catalyze the two electron oxidation of paracetamol to a highly reactive molecule, N-acetyl-*p*benzoquinone imine (NAPQI). The exact CYP isozymes involve in paracetamol oxidation varies and depends upon the concentration of the drug. In human liver microsomes CYP2E1, CYP1A2, CYP3A4, and CYP2D6 are reported to be involved in the conversion of high doses of paracetamol to NAPQI.

Detoxification of NAPQI occurs by conjugation of NAPQI to GSH (an endogenous antioxidant molecule) via enzymatic reaction catalyzed by glutathione-S-transferases (GSTs), yields a GSH conjugate, [3-(glutathione-S-yl)-acetaminophen] and an oxidation product, namely glutathione disulfide (GSSG). The final detoxified product is ultimately excreted in the urine as cysteine and mercapturic acid conjugates.[16-21] The detoxification of NAPQI is extremely rapid, and the

rapid rate may explain why covalent binding to proteins is not observed in hepatocytes until glutathione is almost completely depleted. In healthy condition GSH can be regenerated from GSSG by the enzyme glutathione reductase (GSR). In conditions such as acute paracetamol overdose, the activity of GSR may be inhibited and the level of GSH in the cell is insufficient to detoxify all the NAPQI molecules. The resulting depletion of GSH enables NAPQI to react with protein sulfhydryl groups of cysteine, causing damage to cellular proteins and disturbs their functions.

Further, McGill *et al.*[22] reported the elevated levels of biomarkers of mitochondrial damage such as glutamate dehydrogenase [GDH] and fragmentation of mitochondrial DNA and nuclear DNA in the plasma of paracetamol overdose patients. Masubuchi *et al.*.[23] observed that binding of NAPQI to mitochondrial proteins leads to mitochondrial oxidative stress due to over production of free radicals and this causes the mitochondrial membrane permeability transition (MPT), pore opening, matrix swelling and outer membrane lysis in rodent model. These results suggest that mitochondrial damage and nuclear DNA fragmentation are likely to be the critical events in paracetamol hepatotoxicity and resulting in necrosis of liver tissues. Loss of liver function leads to chronic "hepatic encephalopathy" and may cause irreversible brain damage and multi organ failure. Currently, the only effective therapy for paracetamol overdose is GSH replacement in order to scavenge NAPQI, which is accomplished with N-acetyl-l-cysteine (NAC) and other sulfhydryl donors. However, NAC needs to be given within 12 to 24 hours of paracetamol ingestion.[24]

The events of molecular mechanism of paracetamol toxicity in the liver can be further summarized as follow:

i) Metabolism of paracetamol by cytochrome P_{450} enzyme system produces NAPQI, a reactive metabolite, which depletes glutathione (GSH) pool and covalently binds to proteins.

ii) Loss of GSH with an increased formation of radicals (such as reactive oxygen species and reactive nitrogen species-ROS and RNS) in hepatocytes and such condition induce oxidative stress.

iii) Increased oxidative stress associated with alterations in calcium homeostasis and initiation of signal transduction responses causes transition in the mitochondrial permeability.

iv) Mitochondrial permeability transition occurring with additional oxidative stress and loss of mitochondrial membrane potential results in loss of the ability of the mitochondria to synthesize ATPs.

v) Loss of ATP pool will lead to further cell death and necrotic damage in the liver tissues. (Fig. 2)

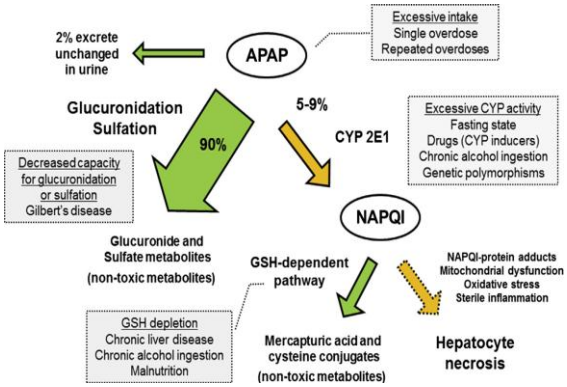

Fig. 2: Molecular mechanism of liver injury induced by paracetamol.[12]

ALCOHOL

The term alcohol originally refers to the primary alcohol (ethyl alcohol or ethanol), and it is obtained by microbial (yeast) fermentation using glucose from carbon sources such as sugar, starch and/or other carbohydrates. Ethanol is an organic compound and its molecular formula is C_2H_6O.

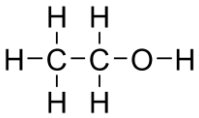

Fig. 3: Chemical structure of alcohol

Alcohol has a long history of several uses worldwide. It is used as fuel and it has many scientific, medical, and industrial uses also. It is the main ingredient in alcoholic beverages. An alcoholic beverage is a depressant which in low doses causes euphoria, reduce anxiety and in higher doses causes intoxication (drunkenness), stupor and unconsciousness. Since drinking alcohol plays an important social role in many cultures, most countries have laws to regulate its production, sale, and consumption. In some of the countries alcoholic beverage is referred as an entertainment food. Long term use (alcoholism) can lead to alcohol abuse and physical disabilities and capable of damaging nearly every organ and system in the body, particularly the brain, heart, liver, pancreas, and immune system.[25] The damage of liver cells by alcohol is mainly by inducing lipid peroxidation and oxidative stress which may result in hepatitis and cirrhosis.[26]

Molecular mechanisms of liver injury induced by alcohol

Since liver is being situated at a point adjacent to the digestive system, toxic substances including alcohol from digestive tract are brought through portal vein to liver before they reach other organs, particularly the heart and brain. Alcohol is a small two carbon molecule and due to its small size it is soluble in both aqueous and lipid environments. This allows alcohol to freely pass from body fluids into cells. Since the portal circulation from the gut passes first through the liver, the bulk of ingested alcohol is metabolized in the liver. The liver is the only organ that can dispose significant amount of

alcohol (80%), but its maximum ability of clearance is fixed. Long term use of alcohol in excessive quantities leads to the development of alcoholic liver disease (ALD). The process of alcohol oxidation involves at least three distinct enzymatic pathways. The first pathway involves two main enzymes which are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). In the first step, ADH metabolizes alcohol (C_2H_6O) to acetaldehyde (C_2H_4O), a highly toxic molecule. Then in the second step, acetaldehyde is further metabolized to another less active molecule called acetic acid ($C_2H_2O_2$) by ALDH, which is further metabolized to water (H_2O) and carbon dioxide (CO_2) and they can be easily excreted.[26]

The complete chemical reaction is: $C_2H_6O \rightarrow C_2H_4O \rightarrow C_2H_2O_2 \rightarrow$Acetyl CoA$\rightarrow 3H_2O+2CO_2$

The second pathway involves in alcohol metabolism is cytochrome P4502E1 (CYP2E1) system and it is a major contributor to the so-called Microsomal Ethanol Oxidizing System (MEOS). MEOS is active when a person has consumed large amount of alcohol. CYP2E1 generates ROS such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) during its catalytic cycle. In the presence of iron, which is increased after ethanol treatment, more powerful oxidants including ·OH, ferryl species, and 1-hydroxyethyl radical are produced. Initially, the liver cells respond to the CYP2E1-related oxidative stress by transcriptionally inducing antioxidant enzymes via their antioxidant response elements. Ultimately, these protective mechanisms are overwhelmed, and the cells become sensitive to the CYP2E1-generated oxidants. These various oxidants can promote toxicity by protein oxidation and enzyme inactivation, oxidative damage to the DNA, and disturbing cell membranes via lipid peroxidation and production of reactive lipid aldehydes, such as malondialdehyde (MDA), hydroxyethyl radical (HER), and 4-hydroxy-2-nonenol (HNE). Mitochondria appear to be the critical cellular organelles damaged by CYP2E1-derived oxidants. A decrease of mitochondrial membrane potential and perhaps the mitochondrial membrane permeability transition causes release of pro-apoptotic factors, resulting in apoptosis. Some CYP2E1-derived reactive oxygen species, such as H_2O_2, lipid hydroperoxide (LOOH) and HNE are diffusible and may exit hepatocytes and enter other liver cell types such as stellate cells and stimulate these cells to produce collagen and elicit fibrosis. [27]

The third pathway involves a non-oxidative pathway catalyzed by fatty acid ethyl ester synthase and this pathway results in the formation of fatty acid ethyl esters (FAEEs). Oxidation of alcohol can also occur in peroxisomes by the activity of catalase. However this oxidation pathway requires the presence of hydrogen peroxidase generating system and it plays no role in alcohol metabolism in normal physiological conditions and further catalase metabolizes only a small amount of alcohol in the liver. CYP2E1 is induced by chronic alcohol consumption and assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. Since acetaldehyde is approximately 30 times more toxic than alcohol, and it is the major causal agent for the alcohol associated ill effects.[28] Even though acetaldehyde exists in the body only for a short time before it is further broken down into acetic acid and then to carbon dioxide and water, it has the potential ability to cause significant molecular damage in the liver tissues by producing large amount of toxic oxidative free radicals (ROS) such as superoxide anion radical, hydrogen peroxide, and hydroxyl radicals.[29] Normally, ROS can be eliminated by *in vivo* antioxidants, particularly glutathione (GSH). When cell's antioxidants are depleted and ROS accumulation reaches a critical threshold the mitochondrial damage will be severe. This process leads to the release of cytochrome c from the mitochondria, which then activates caspases apoptotic pathway (Fig. 3).[30] Further, the accumulation of ROS creates oxidative stress and damage to cellular molecules such as lipids, proteins, and nucleotides and cause death of hepatocytes.[31]

Two biochemical perturbations resulting from oxidative stress due to alcohol toxicity are lipid peroxidation and oxidation of proteins (formation of protein carbonyls).[32] The lipid peroxidation process influences membrane fluidity as well as affect the integrity of biomolecules associated with the membrane (membrane bound phospholipids and proteins).[33] Proteins constitute the major 'working force' for all forms of biological activities. Their exact pattern of folding are directly related to their activity and function. Reactive nitrogen species (ROS and RNS) are formed in higher fluxes under pathogenesis due to alcohol injury that can cause the carbonylation of amino acid residues (oxidation) of proteins and produce protein carbonyls. Protein carbonyl content is the most widely used marker to measure oxidative damage of proteins, which was found higher in alcohol toxicity.[34,35] Lipid peroxidation speeds up the accumulation of fatty acids by breaking down the phospholipids in the liver cells. Acetaldehyde is oxidized to acetate by mitochondrial acetaldehyde dehydrogenase (ALDH2) when it enters in to the mitochondria. Large amount of acetate generation can also be a significant contributor to overall pool of acetyl CoA, which is utilized as precursor for fatty acid and cholesterol biosynthesis. If the condition persists, alcohol interacts with fatty acids and form fatty acid ethyl esters (FAEEs) and this is the 1st stage of liver damage, called Fatty Liver Disease (FLD) or Fatty Liver Syndrome (FLS). Further, FAEEs inhibit the availability of nutrients and oxygen to the liver cells and if the condition exists to a considerable period, cells are accumulated with extracellular matrix, including collagen and produce fibrous scars in the liver tissues. This is the 2nd stage of liver damage called fibrosis (cellular hardening). Fibrosis is reversible with abstinence from alcohol consumption and supplement of good nutrition, but the 3rd stage (last

stage), known as cirrhosis is not reversible. Cirrhosis is a serious liver disease marked by inflammation and damage of cell membrane of hepatocytes and preventing detoxification of toxic molecules in the cells, resulting in scarring and necrosis (cell death). To date, liver transplantation is the only curative treatment for liver cirrhosis. It is one of the major health problems in developed countries and it is the 4th leading cause of death in the American adults and about 70% of death is associated with alcoholism.[36]

The other impacts of alcoholism on hepatocytes are mitochondrial dysfunction, decreased methylation capacity, endoplasmic reticulum stress, impaired vesicular movement and altered proteasome pathway.[37] The occurrence of apoptotic bodies generated from hepatocytes activates Kupffer cells (hepatic macrophages), [38] and the activation of Kupffer cells is the early response for the alcohol-induced liver injury.[39] These inflammatory mediators are responsible for the dysfunction of hepatocytes and leads to apoptosis.[40]

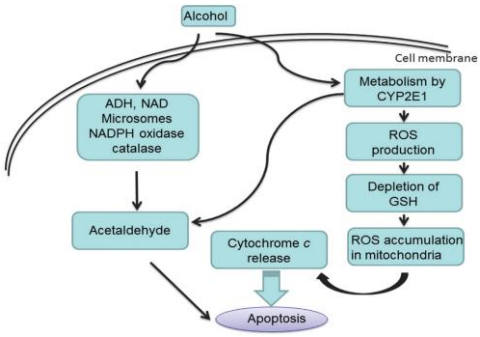

Fig. 4: Molecular mechanism of alcohol-mediated hepatocyte apoptosis.[30]

CARBON TETRACHLORIDE (CCl_4)

Carbon tetrachloride, (also known as tetrachloromethane) is an organic compound with the chemical formula of CCl_4. It is a colorless, organic solvent with a sweet smell that can be detected even at lesser quantity. CCl_4 is used as fuel additive, catalyst, refrigerant (manufacture of chlorofluorocarbons) or metal cleaning agents, adhesives, paints and in fire extinguishers. Currently, usage of CCl_4 in commercial applications has been declined because of the awareness of its health risk. The other sources of CCl_4 are from marine algae, oceans, volcanoes and drill wells and the majority of CCl_4 in the environment is released into air during production or disposal of the compound.[41]

The residual CCl_4 is discharged into soil with other wastes associated with its production. Members of the general population are most likely to be exposed to CCl_4 through ambient air and drinking water. Despite its ban from consumer products, the long lifetime of CCl_4 in the atmosphere contributes to the residual level to which the general population is exposed.[42] Exposure to CCl_4 may also occur by dermal and inhalation routes while using contaminated tap water for bathing and other household purposes.[43] Exposure to CCl_4 via food is not likely to be significance, since its level in most food products is below analytical detection limits. Ingestion of bread or other products made from CCl_4-fumigated grain may contribute to dietary exposure in the past, but this route of exposure is no longer believed to be of significance.

In the drug development field, CCl_4 is widely used to treat animals to create *in vivo* model for liver injury. Damage by CCl_4 is regarded as the analogue of liver damage caused by a variety of hepatotoxins in humans and hence it is widely used in scientific research to evaluate hepatoprotective agents. Toxicity reports on CCl_4 confirm that it is a hepatotoxin and prolonged exposure might result in damage to the central nervous system and kidneys. Symptoms of high exposure include general gastric problems, anorexia, headache, depressive symptoms and giddiness.[44]

Molecular mechanisms of liver injury induced by CCl_4 toxicity

CCl_4 is one of the xenobiotics and it is reported to induce acute and chronic liver tissue injuries.[45] CCl_4 is metabolized by cytochrome P4502E1 (CYP2E1) enzyme system to trichloromethyl radical (CCl_3*). In the presence of oxygen, the CCl_3* radical is further metabolized to the trichloromethyl peroxy radical (CCl_3OO*). Trichloromethyl radical is more important in covalent binding to both lipid and protein molecules, while trichloromethyl peroxy radical initiates peroxidation of polyunsaturated fatty acids. The onset of fat accumulation depends mainly upon haloalkylation, and not upon lipid peroxidation. Some of the damage begins with haloalkylation and lipid peroxidation will cause damage in later stage and both could be involved in the pathogenesis of liver necrosis determining an increased susceptibility of cell to free radicals. Therefore covalent binding by free radicals is implicated in the pathogenesis of cell death during chronic CCl_4 toxicity, whereas acute cell death seems to be mainly depends upon lipid peroxidation. Further, the covalent binding is likely to produce damage at the molecular site where it is produced. On the other hand, lipid peroxidation besides causing loss of membrane integrity, it can also produce toxic metabolites such as 4-hydroxyalkenals and other aldehydes and these radicals can move from the site of origin and produce effect at distant sites. The glutathione (GSH)-dependent mechanism can protect the liver microsomal membrane damage against CCl_4-induced toxicity under aerobic conditions but not under anaerobic conditions. Electrophilic intermediates of free radicals readily react with cellular nucleophiles, primarily with reduced glutathione (GSH) and results in a severe depletion of GSH pool. When the depletion reaches some threshold values lipid peroxidation develops abruptly in an extensive way and this event is eventually cause cellular death. [46,47]. The double allylic hydrogen bonds of polyunsaturated fatty acid (PUFA) are susceptible to the attacks by trichloromethyl peroxide radical and induce an increase in the levels of lipoperoxide and free

peroxide radicals. These radicals induce alterations in the cholesterol profile and decrease the activity of hepatic antioxidant enzymes, in addition to induction of oxidative DNA damage, and induce DNA adducts, strand breakage, chromosomal alterations and genetic mutations.[48]

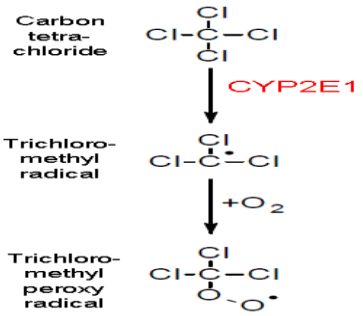

Fig.5: Metabolic activation of carbon tetrachloride (CCl4).[46]

Lipid peroxidation and protein oxidation induced by CCl_4 toxicity

It is well established that lipid peroxidation plays a major role in CCl_4 induced fatty liver disease and cirrhosis.[49] Level of lipid peroxidation is estimated by measuring the malondialdehyde (MDA) content. MDA is formed due to the degradation of polyunsaturated lipids by free radicals. This compound is a reactive aldehyde and is one of the reactive electrophilic species that cause toxic stress in the cells and form covalent protein adducts. MDA was found significantly increased (7 fold-14 h value) upon single exposure to CCl_4 in animal studies.[50,51] Oxidation of protein by CCl_4 intoxication may be an early indication of liver damage. The rate of oxidation of protein is measured by the estimation of Protein Carbonyl content (PCC).[52] A significant increase in the PCC of the liver after exposure to CCl_4 was recorded in an animal study. A greater increase in liver PCC level was recorded in chronic liver injury (approximately 3 fold) than in the acute liver injury (only 2 fold).[53] Even though lipid peroxidation plays a major role in CCl_4 induced liver injury, oxidative damage to mitochondrial DNA is also implicated in the severity of liver injury by CCl_4.[54]

N-NITROSOAMINES

During 1970s, there was an increased frequency of liver cancer found in Norwegian farm animals. The farm animals had been fed on herring meal, which was preserved by sodium nitrite. It was explained that the sodium nitrite was reacted with dimethylamine present in the dried fish powder in the meal and produced nitrosodimethylamine, which was the causal agent for liver cancer.[55] Nitrosamines can cause cancers in a wide variety of animal species; a feature suggests that they may also be carcinogenic to human also. At present, available epidemiological evidence from case-control studies on nitrite and nitrosamines reveal that consumption of preserved fish, vegetable and smoked foods supports liver tumour, gastric and oesophageal cancer.[56]

Nitrosamines are used in the manufacture of some cosmetics, pesticides, and in most of the rubber and latex products.[57] and in certain foods and consumables. In foods, the formation of nitrosamines can occur only under certain environments, including strong acidic condition. Under acidic condition the nitrite compounds forms nitrous acid (HNO_2), which is protonated and splits into the nitrosonium cation (N≡O+) and water. The nitrosonium cation subsequently reacts with an amine and produce nitrosamine. These processes accumulate significant level of nitrosamines in many foodstuffs, especially in meat, fish, and their by-products, and also in cheese products which are preserved with sodium nitrite. Frying of foodstuffs in high temperature can also enhance the formation of nitrosamines. Further, nitrosamines occur in fermented tobacco products and tobacco smoke and this is the most widespread source of human exposure, other than some occupational exposures.

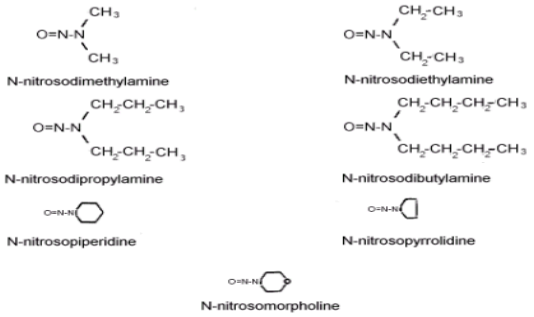

Fig. 6: Chemical structure of different N-Nitrosamine compounds.[57]

Many N- nitrosamines are reported as potent carcinogens, hepatotoxins, mutagens and also immunosuppressive agents. Among the various nitrosamine compounds, N-nitrosodiethylamine (NDEA or DEN) and N-nitrosodimethylamine (NDMA) are identified as highly dangerous hepatotoxins[58]

N-Nitrosodiethylamine

Nitrosodiethylamine (NDEA) is a synthetic compound and its chemical formula is $C_4H_{10}N_2O$. It is a light-sensitive, volatile, yellow colored oil and it is soluble in water, lipids, and other organic solvents. It is used as gasoline and lubricant additive, antioxidant and stabilizer for industrial chemicals. When it is decomposed, nitrosodiethylamine emits toxic fumes of nitrogen oxides. NDEA present in food can be liberated from food during digestion.[58]

N-nitrosodimethylamine

NDMA is the simplest dialkylnitrosamine, with a molecular formula of $C_2H_6N_2O$. NDMA is a potent carcinogen and its chronic exposure results in liver tumours and acute doses cause centrilobular hepatic

necrosis. It may be present in water, air, and soil due to chemical reaction between naturally occurring precursors such as nitrosatable substrates (secondary amines) or nitrosating agents (nitrites). For example, NDMA may form in the air during night time as a result of the atmospheric reaction of dimethylamine (DMA) with nitrogen oxides. Soil bacteria may also synthesize NDMA from various precursors, such as nitrate, nitrite, and amine compounds. NDMA precursors are widespread throughout the environment, occurring in plants, fish, algae, urine, and feces. Concern about NDMA is mainly focused on its presence in food, consumer products, and polluted air. But, currently concern is focused on NDMA as a contaminant in drinking water, resulting from the reactions during chlorination of water or through the direct contamination by industrial wastes.[59]

Molecular mechanisms of liver injury induced by NDMA

Multiple molecular mechanisms are reported to be involved in the metabolism of NDMA and mainly either by *alpha*-hydroxylation or denitrosation resulting in the production of oxidative molecules.[60] and acute single doses of NDMA cause centrilobular hepatic necrosis indicating that metabolism is a major factor in its toxicity.[61] Major route of metabolism (about 67%) *in vivo* is demethylation and this action is catalyzed by cytochrome P4502E1 [CYP2E1] enzyme system.[62] There are two isozymes of CYP2E1 (a low affinity and a high intensity forms) are involved in the first oxidation step, lead to the production of formaldehyde and hydroxymethylnitrosamine. Hydroxylmethylnitrosamine rearranges to yield monomethyl- nitrosamines [$CH_3(CH_2)N–N=O$] and then rearranges to give methyldiazohydroxide, then methyldiazonium ion ($CH_3N_2^+$), and finally methylcarbonium ion ($CH_2CH_2^+$). Both methyldiazonium ion and methylcarbonium ion are highly reactive alkylating agents which will methylate nucleophilic sites of nucleic acids and proteins and impair their function and ultimately results in cell death and necrosis. The degree of methylation of DNA *in vivo* in various tissues correlates with the susceptibility of those tissues to tumour induction. The methylation of DNA is greater in the liver than any other organ including the kidneys.[63] The metabolism of NDMA and the methylation of DNA was studied in male Sprague-Dawley rats. Denitrosation pathway was reported to be involved in the metabolic conversion of NDMA and resulted in the production of methylformaldiamine (CH_3NH_2) in the first step and in the subsequent step methylamine and formaldehyde are generated. [64] It is evident that in both metabolic pathways of NDMA formaldehyde molecules are generated (Fig.7). The US National Toxicology Program (2011) classified that formaldehyde is a "known human carcinogen".

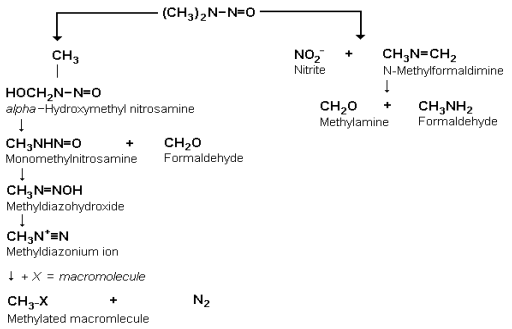

Fig. 7: Metabolic pathway of NDMA.[57].

Further, the intermediate reactive molecules of NDMA are responsible for cancer initiation by inducing DNA adducts and breaking of DNA strands and hepato-carcinogenesis without cirrhosis through the development of putative pre-neoplastic focal lesions. Furthermore, some of the reactive molecules can react covalently with several biomolecules such as protein, nucleic acids and lipids resulting in cellular membrane degradation, increased permeability and leakage of cytoplasmic contents and nutrients from liver cells, which further leads to centrilobular tissue necrosis.[62]

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