CHAPTER-3

REVIEW OF LITERATURE
Metals have been the integral part of human life since from years, some metals referred to as trace elements are essential for normal functioning of the body. The wide spectrum usage of metals from industry, pharmaceutical to daily appliances has increased the susceptibility of their exposure in day today life. As a consequence metal toxicity has become one of the major medical concerns. Exposure to metals resulted in broad range of toxicities at various levels. Metals have also found application in military since 1980’s in the form of depleted uranium (DU), but the use of DU as an anti-armor ballistic material has raised several controversial issues for the environment and related health problems.

Concern regarding the health effects of HMTAs has risen with the discovery that rats intramuscularly implanted with pellets containing 91.1% W, 6% Ni, and 2.9% Co rapidly developed aggressive metastatic tumors at the implantation site (Kalinich et al., 2005). Another study carried out by Miller et al., (2001) showed transformation of neoplastic human osteoblast cells to the tumorigenic phenotype by heavy metal-tungsten alloy particles. The in vivo carcinogenic potential of HMTAs containing W, Ni, and Co is supported by in vitro studies, which demonstrated that exposure to military-relevant mixtures of W, Ni, and Co (WNiCo) induced malignant transformation, generation of reactive oxygen species (ROS), oxidative DNA damage, and expression of several stress genes in various cultured cell types, suggesting a synergistic effect that exceeded the effects of the metals individually (Miller et al., 2002, 2004; Harris et al., 2011). In one of the study carried out by Verma et al., (2011) have shown epigenetic modification triggered by tungsten-alloy exposure in C2C12 (mouse myoblast) and hippocampal primary neuronal cultures, suggesting the underlying synergistic effects of tungsten, nickel and cobalt mediated by changes in intracellular calcium homeostasis and buffering. Roedel and co-worker (2012) have shown that inhalation of both type of HMTAs (WNiCo and WNiFe) causes lung injury by inducing macrophage activation, neutrophilia and the generation of toxic oxygen radicals. They also alter the expression of genes associated with oxidative and metabolic stress and toxicity. Kalinich et al., (2005) indicated the potential for tungsten alloy-induced immunotoxicity (expressed by increased spleen weight and decreased thymus weight) and also the potential for tungsten alloy induced hematotoxicity (expressed by increases in leukocyte and erythrocyte counts, hemoglobin, and hematocrit).
Among the heavy metal found in HMTAs, tungsten and iron are of low toxicity and exposure of these metals by implantation or by inhalation has not been positively associated with long term toxic effects, whereas nickel and cobalt are mainly found to be highly toxic. The shrapnel made from HMTAs, if not surgically removable, may result in similar tumours in humans. Also the occupational exposure to these hard metals may have adverse effects on respiratory tract and also on the skin. Due to the genotoxic, epigenetic and carcinogenic transformation of HMTAs, there is a clear need to develop rapid and robust in vitro methods to characterise the toxicity of HMTAs, in order to identify those that are most likely to be harmful to human health and to guide development of new materials in the future. Very few studies have been carried out to know the toxicity of heavy metal tungsten alloys. Knowing what occurs at the cellular level may help researchers find more ways to counteract the detrimental effects of heavy metal tungsten alloys. NMR based metabonomics has proved to be a very valuable tool in studying biomarkers/metabolic markers identification in various fields. It involves the application of advanced analytical and statistical tools to profile changes in levels of endogenous metabolites in tissues and biofluids resulting from disease onset, stress, or chemical exposure.

Metabolomics allow measuring the multiparametric response of an organism to a stimulus through metabolic fingerprints from biological fluids such as urine or blood plasma/serum using analytical technologies such as nuclear magnetic resonance spectroscopy (NMR) or mass spectrometry (MS). Proton (1H) NMR spectroscopy is uniquely suited to detect a large range of endogenous low-molecular weight metabolites in biological systems as it is rapid, non invasive technique that is rich in structural and quantitative information and allows simultaneous analysis of several metabolites (Nicholson and Wilson, 1989).

The interest was in identifying biomarkers/metabolic markers associated with heavy metal tungsten alloys using NMR spectroscopy. The present proposed work has been focussed on the biochemical application of NMR, involving the analysis of cells, tissue, extracts and body fluids or in vivo application for identification of potential biomarkers, which could further be helpful in designing the strategies for their treatment through metabolomics in human population.
3.1 Mechanism of toxicity of heavy metal used in HMTAs

3.1.1 Role of nickel in HMTAs toxicity

The majority of nickel production is used for the creation of stainless steel, nickel alloys, and nickel cast iron that comprise objects, such as coins, electrical equipment, tools, machinery, armaments, jewellery, and household utensils. Nickel is commonly used in a wide variety of metallurgical processes such as electroplating and alloy production, and in nickel–cadmium batteries, alkaline batteries, dye mordant, catalysts, and electronic equipment. Many nickel compounds are released into the atmosphere during mining, smelting and refining operations (Venugopal and Luckey, 1978). Exposures may take place by inhalation, ingestion or skin contact occur in nickel and nickel alloy production plants as well as in welding, electroplating, grinding and cutting operations. Non-occupational sources of nickel exposure include food, air and water.

Nickel and its compounds have been reported to be potent carcinogenic and/or toxic agents in humans and experimental animals (Sunderman et al., 1984; Coogan et al., 1989). Apart from the potential carcinogen, nickel is also known hematotoxic, immunotoxic, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic and hepatotoxic in nature. It is also a potent skin allergen as it causes contact dermatitis. Nickel depletes glutathione and protein-bound sulphhydryl groups, resulting in the production of reactive oxygen species (ROS) as superoxide ion, hydrogen peroxide, and hydroxyl radical. As a consequence, enhanced lipid peroxidation, DNA damage, and altered calcium and sulphhydryl homeostasis occur (Stohs and Bagchi, 1995; Das et al., 2006).

Studies have indicated that ROS generation is one of the main mechanisms in the carcinogenicity of Ni\(^{2+}\) and the cytotoxicity of acute nickel exposure (Beyersmann and Hartwig, 2008; Cangul et al., 2002; Costa et al., 2002; Denkhaus and Salnikow, 2002). Both in vivo and in vitro tests on cells of higher organism showed that nickel produces some genetic abnormalities like DNA strand break, DNA-protein cross links, nucleotide excision, single gene mutations, sister chromatid exchanges, micronuclei, nucleic acid concentration alteration and cell transformation (Coogan et al., 1989; Coasta, 1991; Das and Dasgupta, 2000). Kidney serves as a major organ of nickel excretion and is a target organ for acute nickel toxicity owing to nickel accumulation and the toxicokinetics of nickel (Sunderman, 1988). Toxic nephropathy with proteinuria and aminoaciduria along with altered proximal tubules and glomeruli histology have been demonstrated during acute nickel toxicity (Gitlitz et al., 1975). Changes at the
metabolites level in renal tissue and urine were seen after acute toxicity of \( \text{NiCl}_2 \) through metabolomics. Also neurological effects (giddiness, weariness) were reported in individuals accidentally exposed to nickel and boric acid in drinking water (Sundermann et al., 1988). Neurological signs (lethargy, ataxia, prostration) were observed in dying rats treated with nickel for 3 months; however, these effects were probably associated with overall toxicity (ATSDR, 1988)

3.1.2 Role of cobalt in HMTAs toxicity
Cobalt is a microelement present in almost all the animals and organisms. Its biological importance is due to its essential role in the formation of vitamin \( B_{12} \) and others cobalamines. Cobalt, a known heavy metal also has multiple industrial applications such as preparation of high strength steels, magnetic alloys, and corrosion-resistant alloys for aeronautical applications. It is also used in electroplating to prepare paints and batteries. The occupational cobalt exposure occurs in cobalt processing plants, hard-metal industry, diamond polishing and ceramic industry such as cobalt blue dyes (Christensen, 1995). The use of cobalt–chromium hard-metal alloys in orthopedic joint replacements has created a new source of cobalt exposure.

The mechanism of cobalt toxicity is not clear, but some of the effects of cobalt could relate to its high affinity for sulphhydryl groups, which may cause inhibition of crucial enzymes, e.g. in mitochondrial respiration (Barceloux, 1999); Cobalt can mimic or replace \( Mg^{2+} \), \( Ca^{2+} \) and other ions in many reactions (Jennette, 1981) and also generates ROS in cells, e.g. by Fenton-like reactions, causing oxidative stress resulting in oxidative damage to DNA, proteins and lipids in several tissues (Maxwell and Salnikow, 2004; Petit et al., 2005; Ahmed and Siddiqui, 2007). Perhaps, the most important effects of cobalt could be caused by metal-induced activation of hypoxia-inducible factor (HIF) thereby promoting tumor development and growth in vivo. It also has an effect on the genes encoding for angiogenic growth factors and angiogenesis, glucose transporters, an array of glycolytic enzymes, and coding for regulation of apoptosis/cell proliferation (Maxwell and Salnikow, 2004, Ke and Costa, 2006). Cobalt is known to be genotoxic as well as carcinogenic, it causes DNA breakage and inhibition of DNA repair mechanism (Lison et al., 2001, Anard et al., 1997; Hamilton-Koch et al., 1986; Painter and Howard, 1982).

Garouei et al., (2011) have shown the effect cobalt on both liver and kidney of adult rats and their suckling pups. They have found decrease in the antioxidant enzymes
such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduced glutathione (GSH). Also the activity of alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and the level of bilirubin, serum urea and creatinine was found to be increased. Histological studies showed an infiltration of mononuclear cells and vascular congestion in liver. Cobalt inhibits heme synthesis in the liver (De Matteis and Gibb, 1977) and also induces heme oxygenase (Maines and Kappas, 1976), with combined effect of rapidly decreasing cytochrome P₄₅₀ concentration. Smith and Fisher (1973) have reported that cobalt toxicity has an acute effect on renal erythropoietic factor and kidney hydrolase activity i.e., it causes increase in renal erythropoietic factor and plasma erythropoietin. Liu et al., (2010) have reported that cobalt chloride had toxic effects on the heart and liver; however, no significant effect was apparent in the kidney. Also cobalt chloride significantly increased AST, ALT and creatinine kinase (CK) concentrations in serum.

Cobalt is also suspected to cause neurotoxic effects, as indicated by the report of memory deficit among workers exposed to hard metal both as dust powder and in mist form (Jordan et al., 1997). Uptake of cobalt in the nasal mucosa and transport to the olfactory bulbs may be an important route of brain exposure (Persson et al., 2003). Experiments performed in rat have shown that cobalt can cause decreased exploratory behavior (Czarnota et al., 1998) and depletion of neurotransmitters (Hasan et al., 1980). Besides inhibiting synaptic transmission by presynaptic blockade of calcium channels, cobalt can also block postsynaptic responses induced by neurotransmitters in vitro (Gerber and Gahwiler, 1991).

### 3.1.3 Role of tungsten in HMTAs toxicity

Tungsten is used in the manufacture of alloys, in light filaments, in X-ray and electron tubes, in phonograph needles, and in contact points for vehicle, telegraph, radio, and television equipment (Budavari, 1996). Other applications include its use in glass-tometal seals, metal evaporative work, windings and heating elements (in furnaces and vacuum-metallizing equipment), ferrous and nonferrous alloys (e.g., high-speed tool steel), welding electrodes, rocket nozzles and other aerospace applications, shell steel, chemical apparatuses, high-speed rotors (e.g., gyroscopes), solar energy devices, and plating material. Occupational exposure to tungsten compounds is possible from inhalation of dusts and dermal contact during the production or use of
tungsten-containing compounds. Large amounts of tungsten dust are released from the
crushing and milling of ores.

Tungsten and its compounds are toxic at very high doses and very few studies
are reported for the toxicity of tungsten. Liver is one of the major sites for tungsten
distribution in the body (Leggett, 1997; Haneke, 2003), possibly because of the high
concentration of molybdenum-dependent enzymes (e.g., xanthine oxidase [XO]), which
tungstate competes for the binding site, rendering the enzymes inactive (Johnson et al.,
1974 a and b). Tungstate has been shown to polymerize with phosphate, forming
polyphosphotungstates (Koutsospyros et al., 2006; Bednar et al., 2008, 2009), as well
as be incorporated into bone in lieu of phosphates (Lagarde and Leroy, 2002). Several
phosphate pathways are critical to normal homeostasis; thus, it is possible that tungsten
interacts with phosphate-dependent processes in the body. Neurological and chronic
respiratory problems in metallurgy workers, miners, and in a French soldier exposed to
tungsten have been reported (Jordan et al., 1990; Marquet et al., 1996; Chen et al.,
2005). Tungsten exposure via drinking water has been associated with onset of acute
lymphocytic leukemia clusters (Steinmaus et al., 2004; Rubin et al., 2007; Sheppard et
al., 2007). Tungsten is known to alter phosphate dependent biochemical pathways in a
cell-type-dependent manner that may negatively affect downstream cellular functions
(Johnson et al., 2010). Subtle neurobehavioral defects were observed in rats exposed to
sodium tungstate (McInturf et al., 2008).

All the three heavy metal used in HMTAs are found to be toxic at all level. All
the metal ions are potent hepatotoxicant, nephrotoxicant and neurotoxicant. Toxicology
aims at studying adverse effects of chemicals (xenobiotics) on living organisms. The
toxicity of a given compound/metal refers to its ability to disrupt some biological
functions at a certain level of biological organization (i.e., cell, tissue, or organ).
Toxicological studies are mainly based on histopathological evaluation and serum or
plasma biochemical examination. Compared with conventional clinical biochemical
tests and histopathological examination, metabolomics has unique superiority (Holmes
et al., 2000, 2001). First, the non-invasive sample collection in metabolomics makes it
an attractive technique for developing exposure biomarkers of chemical toxicity in both
preclinical and subsequent clinical phases (Robertson et al., 2005). Second,
metabolomics can provide more information on toxicity than the conventional
toxicological evaluation methods in measuring changes, such as in the clinical
chemistry parameters in the process of toxicity generation and development.
Metabolomics studies based on small molecules, which are involved in biochemical processes, provide a great deal of information on the status and functioning of a living system caused by genomic and proteomics alteration. The term metabonomics was defined as the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification (Lindon et al., 2004). Metabonomics involves the application of advanced analytical and statistical tools to profile changes in levels of endogenous metabolites in tissues and bio-fluids resulting from disease onset, stress, or chemical exposure (Shockcor and Holmes, 2002). Metabolomic analysis of bio-fluids (i.e., blood, urine, cerebral spinal fluid, etc.) provides a window into the biochemical status of a living organism.

Metabonomic in conjunction to multivariate analysis has proved extremely successful in terms of toxicity profiling, screening, quality control of food and chemical products (Belton et al., 1998; Zamora et al., 2001), evaluation of environmental contamination (Warne et al., 2000), evaluation of drug efficacy (Connor et al., 1997), classification of tumours (Burtscher and Holtas, 2001; Roda et al., 2000), monitoring physiological variation (Holmes et al., 1994) and disease diagnosis (Roda et al., 2000; Burtscher and Holtas, 2001; Griffin et al., 2001; Huoa et al., 2009). In addition to characterizing disease with high sensitivity and specificity, NMR-based metabolomic analysis extended its application in therapeutic interventions and outcome of diagnosis in an efficient way. $^1$H NMR based metabolomics of bio-fluid and tissue samples has been applied to investigate many diseases and disease models including inborn errors of metabolism, tumor categorisation, evaluation of patients of liver and kidney diseases, identification of early biomarkers for neurodegenerative diseases and monitoring of drug overdose cases (Tate et al. 2000; Burtscher and Holtas, 2001). The progress of disease indicated by a complex phenomenon of gene protein and biochemicals that may be best evaluated using samples obtained regularly over an appropriate time course and prompt sample analysis and interpretation. NMR based metabolomic approach utilized tissue, serum/ plasma, cell lines and breathe condensate for the identification of cancerous tissue or cancer progression. High resolution Magic Angle Spin Nuclear Magnetic Resonance (HR MAS NMR) spectroscopy–based metabolomics has proven to be a powerful tool in the assessment of prostate and renal cancerous tissue. A linear
discriminant analysis (LDA) was performed to distinguish normal and kidney cancer tissue with 100% accuracy (Tate et al., 2000). Even in kidney cancer, normal cortex and medulla were compared with cancerous tissue of similar origin, which showed lower organic osmolyte and higher lipid concentration in renal carcinoma cells, while in papillary renal carcinoma cells, the taurine concentration was higher and lipid signal was absent.

Viant et al., (2005) used NMR spectroscopy to study the early metabolic disturbances following traumatic brain injury in a mammalian model. The metabolic profile of different part of brain tissue extract (hippocampus, cortex) as well as plasma after an hour of brain injury was studied. NMR based biomarkers can also be identified for apoptosis. Lyng and his coworkers (2007) performed the metabolic mapping for assessment of apoptosis in cervical carcinoma cells using high-resolution magic angle spinning (HR MAS) NMR spectroscopy. Investigators showed that onset of apoptosis is accompanied by more than two fold increase in intensity of methylene resonance (at 1.3 ppm) of the membrane lipids. Increase in CH$_2$ resonance signal intensity is parallel to the surface expression of phophatidylserine (an early marker of apoptosis) exposed to the cell surface during apoptosis.

High resolution $^1$H NMR spectroscopy of tissues is not only useful in disease diagnosis, but can also provide complimentary data for use in toxicological screening when coupled with appropriate chemo-metric analysis of drug in vivo. In combination with the multivariate analysis complex, biological profiles generated from spectroscopic technology has enabled the construction of successful expert systems for toxicity screening and disease diagnosis (Henry, 2002). In recent time, the use of HR MAS NMR spectroscopy, in conjunction with diffusion and relaxation weighting has become a technique of choice to probe the metabolic compartmentation within the range of tissues.

Acute or chronic effect of different drugs or toxic elements was extensively studied by NMR spectroscopy on tissue extract as well as using HR-MAS spectroscopy. Wang et al., (2006) used NMR based metabonomics study to investigate the hepatotoxic effects of indole-2 ketone (Z24) on rats, and found high levels of lactate, glutamine together with decrease in glucose, glycogen and choline by metabolic profiling on aqueous soluble extracts of liver tissue. There are few studies, which have optimized sample preparation procedures and biochemical stability of tissue during spectral acquisition by high-resolution magic angle spinning and proton NMR spectroscopy of
intact liver and kidney (Waters et al., 2000; Wu et al., 2008). Tesiram et al., (2005) showed the application of $^1$H NMR spectroscopy in the study of lipid metabolites in rat hepatocarcinogenesis model, as altered lipid metabolism in the liver is a key feature of developing liver nodules and tumors. Similarly idiosyncrasy like liver toxicity in rats by treatment with ranitidine (histamine-2 receptor antagonist) and lipopolysaccharide (inflammagen) studied by NMR based metabonomic profiling (Maddox et al., 2006). This suggests that metabonomic evaluation might be a more sensitive marker of the potential for idiosyncratic drug toxicity than increase in serum liver enzyme activity. Constatinou and his co workers (2007) showed the alteration in hepatic tissue biochemistry in rats after thioacetamide treatment by using $^1$H NMR spectroscopy. Thioacetamide (TAA) is a potent hepatotoxin that causes centrilobular necrosis and nephrotoxic damage and prolonged exposure can result in bile duct proliferation and liver cirrhosis (multi step process resulting from the chronic effect of noxious elements of different nature). Biotransformation of TAA precedes oxidative damage associated liver injury. Liver cirrhosis is considered as a progressive fibrotic process characterized by excess extracellular matrix deposition in the liver with scar formation and destruction of the normal liver architecture. TAA administration reflects increase in lactate as well as acetoacetate and acetate and a decrease in fumarate and increase in succinate in liver could be related to the inactivation of succinate dehydrogenase in Krebs cycle. Disturbed protein synthesis in the liver in addition to protein degradation induced by necrosis would lead to increased concentration of free amino acid in liver and blood. Similarly, HRMAS $^1$H NMR spectroscopic and PR based methods were applied to study the acute biochemical effects of La(NO$_3$)$_3$ on rats (Wu et al., 2005). Study showed that male wistar rats treated with various doses of La(NO$_3$)$_3$ showed increase in triglyceride and bile acid and decrease in liver glycogen, together with slight elevation of triglyceride in kidney tissue. Holmes et al., (2002) showed the effect of cholistatic hepatotoxin α-nepthylisothiocynate (ANIT) on intact liver tissue sample by $^1$H NMR and MAS- NMR spectroscopy and investigated the changes in level of organic acids and bases, amino acids, sugars and glycogen together with bile acids, choline and phosphocholine. The $^1$H NMR-PCA analysis allowed direct detection of all the ANIT induced tissue perturbations revealed by $^1$H NMR of extracts, enabling characterization of the lesions, which included steatosis, bile duct obstruction and altered glycogen / glucose metabolism.
The acute or chronic effect of drugs or toxic elements on kidney tissue was extensively studied using NMR/HR-MAS spectroscopy. Garrod et al., (1999) used HR-MAS to investigate the biochemical composition of normal renal cortex and renal papilla samples from rats, and results were compared with those from conventional $^1$H NMR analysis of tissue extracts. They detected high levels of free amino acids and several organic acids in the cortex, together with choline, glucose, and trimethylamine-N-oxide. New resonances from triglycerides were also shown in the spectra of cortical tissue at higher spinning rate during HR-MAS. Few studies have shown optimised sample preparation procedures and biochemical stability of tissue during spectral acquisition by HRMAS and $^1$H NMR spectroscopy of intact liver and kidney (Waters et al., 2000; Wu et al., 2008).

High-resolution $^1$H NMR spectroscopy is considered as a relatively insensitive technique, which only measures the high-concentration metabolites found in biofluids and intact tissue/tissue extract. Despite this insensitivity, NMR based metabolomics has proven its role in toxicity testing and as a novel diagnostic tool. Moreover, the sensitivity of NMR has increased spectacularly in recent years due to technical advancement such as cryo probes that increase the signal to noise ratio (SNR), small volume sample tubes that allow detection of smaller amounts of sample, improved digital electronics and high power magnets that increase sensitivity of technique several times.

### 3.3 Toxicological metabolomics

In the last two decades, nuclear magnetic resonance (NMR) spectroscopy has been employed in the field of toxicology, and recently NMR spectroscopy-based metabolomics has been extensively exploited in finding potential biomarkers of toxicity and disease (Nicholson et al., 2002; Robertson, 2005; Rantalainen et al., 2006; Zira et al., 2009).

Feng et al., (2002) have used $^1$H NMR based spectroscopy to assess long term toxicological effects of rare earth metal such as La(NO$_3$)$_3$. They have identified numerous metabolites in urine and serum of rats such as creatinine, citrate, glucose, ketone bodies, trimethylamine N-oxide (TMAO), and various amino acids. The increase in the amounts of the excreted ketone bodies, amino acids, lactate, ethanol, succinate, TMAO, dimethylamine, and taurine and a decrease in citrate, glucose, urea,
and allantoin were indicative of liver and kidney damaged due to La(NO$_3$)$_3$. Lin et al., (2009) using LC-MS based metabonomics approach identified 23 endogenous metabolite as biomarkers in urine associated with the hepatotoxicity induced by CCl$_4$. However, these metabolites have been referred as usual suspects as these metabolites change in response to toxicant administration, regardless of the nature of the toxicant, its mechanism of action or its target (Robertson, 2005). Although usual suspects in urine bring challenges in elucidating specific mechanism of a certain toxicant, they do provide a non-invasive measure to general diagnosis of possible toxicology. In addition, usual suspects can be turned into specific biomarkers, if necessary, by the enhancement of mechanistic finding. Jiang et al., (2012) investigated the effect of CCl$_4$ on kidney, lung and spleen using $^{1}$H NMR based metabonomic approach in conjugation with serum clinical chemistry and histopathology. Acute CCl$_4$ exposure caused significant elevation of creatine and decline for glucose, taurine, trimethyl amine (TMA), uridine and adenosine in kidney. In lungs, elevation of amino acids, creatine and betaine together with depletion of glycogen, glucose, taurine, glycine and hypoxanthine was observed. Furthermore, CCl$_4$ caused elevation of lactate, alanine, betaine and uracil with decline for glucose, choline and hypoxanthine in spleen. Lenz et al., (2004) studied the nephrotoxic aspect of mercuric chloride using $^{1}$H NMR and HPLC-TOF/MS metabonomic approach and showed that mercury treatment decreases excretion of creatinine and citrate, while elevating urinary glucose, glycine, alanine, α-ketoglutarate, succinate and acetate. Depletion of both medullar TMAO as well as papillary osmolytes in urine (myo-inositol, betaine) suggested massive damage to all kidney regions (Wang et al., 2006).

Wei et al., (2008) identified the metabolic markers for cinnabar (a traditional Chinese mineral medicine). The metabolic signature of urinalysis exhibited an increase in the levels of creatinine, acetate, acetoacetate, taurine, hippurate and phenylacetylglucine, together with a decrease in the levels of trimethyl-N-oxide, dimethylglycine and Kreb's cycle intermediates (citrate, 2-oxoglutarate and succinate) whereas the analysis of serum showed elevated concentrations of ketone bodies (3-Dhydroxybutyrate and acetoacetate), branched-chain amino acids (valine, leucine and isoleucine), choline and creatine. Boudonck et al., (2009) identified the early biomarkers of nephrotoxicity for nephrotoxins such as gentamicin, cisplatin, or
tobramycin. Using a combination of gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS) a global, nontargeted metabolomics analysis of urine and kidney revealed that increased polyamines and amino acids in urine were early biomarkers of nephrotoxicity prior to observable histological kidney damage and conventional clinical chemistry indications of nephrotoxicity. Upon prolonged dosing, nephrotoxin-induced changes included a progressive loss of amino acids in urine and concomitant decrease in amino acids and nucleosides in kidney tissue. A nephrotoxicity prediction model, based on the levels of branched-chain amino acids in urine, distinguished nephrotoxin-treated samples from vehicle-control samples, with 100%, 93%, and 70% accuracy at day 28, day 5, and day 1, respectively.

Hao et al., (2012) have studied the toxic effect of acephate using metabolomic approach based on ultra-performance liquid chromatography/mass spectrometry (UPLC-MS). They identified metabolites like dimethylthiophosphate and uric acid in urine as sensitive nephrotoxic biomarker of exposure to acephate. In another study carried out by Neerathilingam et al., (2010) the effect of toxic organophosphate, tributyl phosphate (TBP). The $^1$H NMR spectra revealed TBP-induced variations of endogenous urinary metabolites including benzoate, urea, and trigonelline along with metabolites involved in the Krebs cycle including citrate, cis-aconitate, transaconitate, 2-oxoglutarate, succinate, and fumarate. These findings indicated that TBP induced a disturbance to Krebs cycle energy metabolism and provided a biomarker signature of TBP exposure. Apart from the above mentioned endogenous compound, three metabolites of TBP, dibutylphosphate, N-acetyl-(S-3-hydroxybutyl)-l-cysteine and N-acetyl-(S-3-oxobutyl)-l-cysteine are the most important.

$^1$H NMR based metabolomics was applied to study the toxic effect of metal mixtures. Combination of toxicogenomics, proteomics, and metabolomics will be very helpful since these techniques can examine many genes or proteins and interrelated metabolic pathways at the same time.

Wu and Wang (2010) studied the effect of copper (Cu) and cadmium (Cd), both as single metal and as a mixture in marine mussel Perna viridis. The major metabolite changes corresponding to metal exposure are related to amino acids, osmolytes, and energy metabolites. For the Cu, Cd co-exposed mussels, the levels of lactate, branched chain amino acid, succinate, and NAD increased, whereas the levels
of glucose, glycogen, and ATP/ADP decreased, indicating a different metabolic profile for the single metal exposure groups. However, the metabolic profile induced by the metal mixture was very similar to that from Cu exposure, suggesting that Cu dominantly induced the metabolic disturbances. Lu et al., (2010) by using metabonomic approach investigated the effect of environmental endocrine disruptors (EDs), polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alone or in combination, to explore the hepatotoxic mechanisms of PCBs and TCDD exposure. The loading plot of PCA revealed remarkable increases in the level of lactate, glucose, taurine, creatine and 2-hydroxy-isovaleric acid and reductions in the levels of 2-oxoglutarate, citrate, succinate, hippurate and TMAO in rat urine after exposure. These changes were more striking in the combined group. The changed metabolites may be considered possible biomarker for hepatotoxicity. Hong et al., (2004) and Nordberg et al., (2005) utilized a suite of renal proteinuria biomarkers to study groups of persons in China with combined exposures to Cd+As relative to controls and to groups with primarily Cd or As exposures. They observed that coexposure to these elements produced more severe renal effects than exposure to either element alone, demonstrating the value of molecular biomarkers in delineating interactive nephrotoxic effects between these two toxic elements under environmental exposure conditions.

3.4 Magnetic resonance imaging
Magnetic resonance imaging (MRI) continues to have a large impact on the diagnosis and management of a number of diseases, especially associated with brain injury. The strengths of MRI are the unique contrast that can be obtained by altering the parameters which based on different principles viz. T1, T2 based contrast, susceptibility based contrast (T2*), diffusion weighed imaging, perfusion and exogenous contrast. Among diagnostic imaging modalities (CT, X-Ray, PET) MRI is harmless due to non involvement of ionizing radiation and that it can be readily applied to human and animal models. From last two decade due to the technical advancement, a number of tools and techniques are added in the panoply of MRI. Developments of functional MRI techniques that measure aspects of hemodynamics and water diffusion play an important role in the study of brain function. The development of MRI continues at a rapid pace and a renewed push to increased spatial and temporal resolution will extend the applicability of anatomical and functional MRI (Koretsky, 2004).

Identifying an early sign of normal tissue damage with MRI non invasively, would have the potential to predict organ dysfunction, thereby allowing re-optimization of
treatment strategies based upon individual risks and benefits. Early detection with non-invasive imaging may enable interventions to mitigate therapy-associated injury prior to its clinical manifestation (Thorek et al., 2006). Further, successive imaging may provide an objective assessment of the impact of such mitigation therapies. However, many problems make application of imaging to normal tissue assessment challenging, and it is necessary to establish imaging biomarkers as surrogate end points of disease or clinical outcome (Astrakas et al., 2004). Few MRI based techniques, which can detect the pathophysiological response in brain, are summarized in Fig. 3.1.

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<th>MRI Method</th>
<th>Pathophysiology</th>
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<td>• Proton density MRI</td>
<td>Vasogenic edema</td>
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<td>• T1 W MRI</td>
<td>BBB damage</td>
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<td>• T2 W MRI</td>
<td>Tissue degradation</td>
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<tr>
<td>• Contrast-enhanced T1 W MRI</td>
<td>Vasogenic edema</td>
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<td>• BOLD MRI</td>
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<td>• MRA</td>
<td>Disturbed hemodynamics</td>
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Fig. 3.1: MRI methods, pathophysiologic conditions on which these techniques can provide information, and examples of their use in animal models of brain disorders (adapted from Dijkhuizen and Nicolay, 2003).

3.4.1 Diffusion weighted imaging
After two decades of intense research, diffusion weighted imaging (DWI) has progressed from the experimental to the clinical field and may have a major potential impact on imaging diagnosis. Diffusion tensor imaging (DTI) allows tissue structure to be probed and imaged in a microscopic scale, providing clue to the fine architecture of the neural tissue and to changes associated with various physiological and pathological
changes (Le Bihan, 2007). DWI has been extensively studied in both neurological and extraneurological applications including cerebral ischemic stroke (Huisman, 2003; Roberts and Rowley, 2003), tumor grading (Hayashida et al., 2006; Price et al., 2007), differential diagnosis of benign and malignant tumors (Smith et al., 2006; Eida et al., 2007), characterization of viable and necrotic tissues (Koc et al., 2007), assessment of organ functions (Thoeny and Keyzer, 2007), prediction of pathologic outcomes (Khan et al., 2006), and evaluation of tumor response to therapies (Vandecaveye et al., 2006). It is a promising method for characterizing microstructural changes in white matter and has been used in a broad spectrum of CNS applications (Trivedi et al., 2009; Catani, 2006). Wang et al., (2001) studied 81 evaluable lesions (carcinomas, lymphomas, benign salivary gland adenomas and benign cysts) and showed that there was a significant difference in the apparent diffusion coefficient (ADCs) of the four groups of lesions. Only 16% of lesions were not evaluable because of distortion artifacts due to the vicinity of lesions predominantly around airspaces in the paranasal sinuses or larynx. Kamel et al., (2003) studied role of diffusion-weighted imaging in estimating tumor necrosis after chemoembolization of hepatocellular carcinoma. They concluded that diffusion weighted imaging can quantify tumor necrosis to a greater degree than any other imaging modality or even gadolinium-enhanced MRI. Cheung and coworkers (2010) utilized DW imaging to study renal ischemia reperfusion injury (IRI) in Sprague Dawely (SD) rat model. They observed ADC of injured renal cortex to be significantly lower than that of contralateral intact cortex. Meanwhile both ADC and FA of IR-injured medulla were significantly less than those of contralateral intact medulla. The study concluded as DTI can probe both structural and functional information of kidneys following renal IRI. Wang and Fei, (2010) examined the role of photodynamic therapy in subcutaneous tumor xenografts of human prostate cancer cells (CWR22) in athymic nude mice using DW imaging. The average ADC of the 24 treated tumors increased 24 h after photodynamic therapy comparing with control and well corroborated with prostate specific antigen level, and tumor histology.

The heavy metals used in HMTAs are neurotoxic in nature. Tungsten compounds when incorporated as embedded shrapnel frequently cannot be removed from brain tissue because of their location and/or small size, which may results in chronic health effects. Various neurological symptoms have been reported in metallurgy workers, miners, and in a French soldier exposed to tungsten (Jordan et al.,
Depleted uranium which has been replaced by HMTAs is known to cross the blood brain barrier (BBB) and get accumulated in various brain parts of rat showed neurological and neuropsychological symptoms. Since all the heavy metals used in HMTAs are also known to cross the BBB thus causing neurotoxicity. DTI a non-invasive technique, which estimate the microstructural changes occurring in the brain tissue and also provide quantitative information regarding the integrity and orientation of white matter tracts in the brain. It is a unique technique for assessing important white matter pathology in the brain. There are few clinical studies which have shown the potential of the DTI technique to study the neurotoxic aspects of heavy metals. Awasthi et al., (2010) have reported that iron concentration influences DTI metrics in deep gray matter nuclei of patient with PKAN (pantothenate kinase–associated neurodegeneration) and their siblings with age matched control. An increase in FA value of substantia nigra (SN) and globus pallidus (GP) from controls to the patient group to siblings was observed. In the GP, MD values were significantly higher in patients compared with controls and siblings. The patients showed significantly increased FA with decreased MD in hypointense compared with hyperintense regions of the eye-of-the-tiger sign. High FA values in siblings of patients with PKAN suggest the presence of abnormal iron in deep gray matter nuclei. Pfefferbaum et al., (2010) have studied the effect of age and iron concentration on deep gray matter brain structures. The effect of age on the diffusion measures in the deep gray matter structures was distinctly different from that reported in white matter. In contrast to lower anisotropy and higher diffusivity typical in white matter of older relative to younger adults observed with DTI, both anisotropy and diffusivity were higher in the older than younger group in the caudate nucleus and putamen; the thalamus showed little effect of age on anisotropy or diffusivity. The differential effects of age on DTI metrics in subcortical gray matter structures compared with white matter tracts appears to be related, at least in part, to local iron content, which in the elderly of the present study was prominent in the field dependent relaxation rate increase (the (FDRI) estimate) of the putamen and visibly striking in the diffusion-weighted image of the basal ganglia structures. Brubaker et al., (2009) assessed the effect of childhood exposure of lead on white matter tract on adult brain and showed FA values were decreased throughout the white matter region. Regions of the corona radiata demonstrated highly significant lead-associated decreases in FA and AD and increases in MD and RD. The genu, body, and splenium of the corpus callosum demonstrated highly significant lead-associated
decreases in RD, smaller and less significant decreases in MD, and small areas with increases in AD. This study indicates that childhood lead exposure is associated with a significant and persistent impact on white matter microstructure and the changes in the FA and MD values are suggestive of altered myelination and axonal integrity. Thus DTI based imaging modality can be a valuable technique to study the neurotoxic aspects of metals used in HMTAs.