CHAPTER-4

NMR BASED METABOLOMICS
AFTER NiCl₂ INDUCED TOXICITY
4.1 Introduction

Metabolomics measures 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 NMR or MS. Now day’s NMR- and MS-based metabolomics are extensively used for evaluating the biochemical consequences of toxic action of heavy metals resulting in the identification of several metabolic markers. One major strength of metabolomics is that metabolic biomarkers are more readily applied across species than transcriptomic or proteomic biomarkers. $^1$H NMR spectroscopy is uniquely suited to detect a large range of endogenous lowmolecular 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 high dimensionality and complexity of $^1$H NMR metabolomic data requires multivariate statistical techniques for extraction of underlying biological information. Multivariate analysis can simplify the NMR data and aid in identification of target sites of toxicity, assessment of processes of a toxic lesion and characterization of novel biomarker combinations (Lindon et al., 1999; Xu, 2004). In recent years it has proven a valuable tool for interpreting the quantitative relationship between NMR data and a given biological phenotype (Trygg and Wold, 2002; Trygg et al., 2007). Metabolomics data can be employed to study a variety of analytical problems including the potential to classify organ-specific toxicity by comparison with known toxins/toxic compounds. The use of peripheral fluids such as urine for metabolomics analyses makes it feasible to follow individual animals over time after toxic injury (Holmes et al., 1992; Khan et al., 2011; Tyagi et al., 2011) and enables the severity of the injury to be assessed in terms of recovery time. The utility of metabolomics for classifying nephrotoxins such as cadmium chloride (Griffin et al., 2001), mercury chloride (Holmes et al., 2000), gentamicin (Lenz et al., 2005) and cisplatin (Portilla et al., 2006) has been reported.

Nickel, a known heavy metal, has a wide variety of industrial applications and is also known to have adverse effects on human health. Many nickel compounds are released into the atmosphere during mining, smelting, and refining operations (Venugopal and Luckey, 1978). Toxicity studies conducted in mammals have shown that nickel, targets a number of organs and produces multiple toxic effects. Since kidney serves as a major organ of nickel excretion and is a target organ for acute nickel toxicity due to nickel accumulation, hence it has a principal role in the toxicokinetics of nickel (Sunderman, 1988). Nickel affects kidney metabolism by reducing renal clearance rates and also
causes proteinuria. The primary causes for nickel induced toxicity in various tissues, though still under investigation, has been suggested to be the formation of free radicals in various tissues which leads to modifications of DNA bases, enhanced lipid peroxidation (LPO), altered calcium and sulphydryl (-SH) homeostasis (Das et al., 2008). Although traditional analytical approaches have elucidated the toxicity of nickel at biochemical, enzymatic and genetic levels, the associated metabolic alterations remains to be systematically investigated by metabolomics. A holistic approach would be useful to answer these questions and to further our understanding of metabolic effects of nickel chloride (NiCl₂) toxicity. The newly established metabolomic technology is a promising tool as indicated by the investigations into the metabolic response of biological systems to many toxins as well as the byproducts of toxin metabolism (Boudonck et al., 2009). The present study, deals with NiCl₂ induced metabolomic changes in rat urine using a ¹H NMR based metabolomic approach coupled with clinical chemistry information. The objectives were to investigate the multiple metabolic responses of rats to NiCl₂ exposure and to further identify metabolomic phenotypes for nickel induced nephrotoxicity. In addition to this in order to monitor the acute biochemical changes in kidney tissue, the ¹H NMR spectroscopy of kidney tissue extract was also performed. Apart from metabonomic analysis, kidney tissues were analysed for antioxidants enzymes and histopathological alterations in order to correlate the metabolite changes with kidney tissue damage.

4.2 Materials and methods

4.2.1 Chemical and reagents
All chemicals and solvents viz. trimethylsilyl-2, 2, 3, 3- tetradeuteropropionic acid sodium salt (TSP), deuterium oxide (D₂O), phosphate buffer, perchloric acid (6%), disodium hydrogen phosphate (Na₂HPO₄), sodium dihydrogen phosphate (NaH₂PO₄), ethylene- di-amine-tetra acetic acid (EDTA), sodium chloride (NaCl), sodium lauryl sulphate (SLS), sodium citrate, acetic acid, potassium hydroxide (KOH), metaphosphoric acid, 5,5” dithio- bis-2 nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), malonyldialdehyde (MDA), tricarboxylic acid (TCA), hydrogen peroxide (H₂O₂), pyrogallol, haematoxyline, eosin, ethanol, xylene, formalin, acetone, canada balsam and NiCl₂ used in the study were obtained from Sigma- Aldrich (St Louis, MO, USA).
4.2.2 Animals
A total of 50 male Sprague-Dawely (SD) rats, 11 weeks of age (250 ± 20 g) obtained from animal facility of the institute, were acclimatized for 7 days in polypropylene cages and transferred into individual metabolic cages. Food and tap water were provided ad libitum. The room was well ventilated, and the conditions were controlled at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%. Rats were randomly divided into 3 groups (n = 15 in each group) by marking them with picric acid and injected with NiCl$_2$ intraperitonealy (i.p.) prepared in saline at a dose rate of 4 (low dose, one fifth of LD$_{50}$ of NiCl$_2$), 10 (moderate dose, three fifth of LD$_{50}$ of NiCl$_2$), and 20 mg/kg body weight (b.w.) (high dose, LD$_{50}$ of NiCl$_2$) respectively. The dose selected was according to LD$_{50}$ of NiCl$_2$ by i.p. route in rats as reported earlier in material safety data sheet (MSDS, 2006 a). Controls rats (n = 5) were injected i.p. saline only. All animal handling and experimental protocols were approved and confirmed to the guidelines stipulated by the Institutional Animal Ethical Committee.

4.2.3 Collection of urine
For urine collection, animals (n = 5 in each group) were randomly picked from all three NiCl$_2$ treated group and controls and kept in metabolic cages for three days for acclimatization. Urine samples from all the three NiCl$_2$ treated groups were collected in ice-cooled tubes containing 1% sodium azide at 8, 16, 24, 72 96 and 120 h post dose (p.d.) whereas, for control rats (n = 5) urine samples were collected only once throughout the study. To reduce contamination, rats were individually placed in clean cages and 0.1% sodium azide was added in urine. Urine samples were transferred from metabolic cages to eppendorf tube (2.0 ml) and were placed at -80°C until NMR analysis.

4.2.4 Collection of serum and kidney tissue
Animals (n = 5 in each group at each time point including controls) were sacrificed at 24, 72 and 120 h p.d. by cervical dislocation and approximately 1 ml of blood and both the kidneys were collected. Blood samples were allowed to clot at room temperature for approximately 30 mins and subsequently centrifuged for 15 min at 2520 xg. After centrifugation serum was taken with the help of pipette. Blood sample (0.1 ml) was collected in EDTA containing vacutainer for total blood glutathione (GSH). The collected serum samples were placed at -80°C until biochemical or NMR analysis. Both the kidneys obtained from low and high dose NiCl$_2$ treated group by sacrificing them at 24, 72, and 120 h p.d., were rinsed with 0.1 M phosphate buffer
saline (PBS 1:9) to remove trace amount of blood and fatty tissue. One whole kidney was fixed in 10% formalin for histopathological examination and another of kidney was divided into two halves, one of which was immediately snap frozen in liquid nitrogen and stored at -80°C for perchloric acid extraction for $^1$H NMR spectroscopic analysis and another half of kidney tissue was used for antioxidant assay analysis viz. lipid peroxidation (LPO) assay, GSH and superoxide dismutase (SOD) assay. Antioxidant assays were performed on the same day of sacrifice of animals.

4.2.5 Histopathology of kidney
Tissue histopathology was performed, in order to ascertain any significant histopathological alterations at cellular level. Kidney tissue was fixed in 10% buffered formalin. The fixed tissues were transferred to a graded series of ethanol (30-100 %) and then cleared in xylene. Once cleared, the tissues were infiltrated in molten paraffin wax in the oven at 58°C. The paraffin-embedded kidney was sectioned with microtome at <5 µm thick sections of tissue. Tissue sections were gently directed on glass slide and then placed it in a hot air oven to remove wax. Tissue samples on glass slide were dipped in xylene solution for 3-5 mins and then transferred into acetone for 3-5 mins. The slides were gently washed with running tap water twice. Haematoxyline was added for 10-15 mins and then washed again under mild running water. Subsequently eosin was added for 1-2 mins and then washed with acetone and xylene 2-3 times each. Tissue sections were mounted with canada balsam and examined under microscope.

4.2.6 Biochemical analysis of serum
Biochemical analysis was performed to see the alteration in serum samples. Liver and kidney function tests were performed on serum samples. Urea, creatinine, serum glutamate-pyruvate transaminase (SGPT), serum glutamate-oxaloacetate transaminase (SGOT) in serum samples were determined by using diagnostic kit from Erba Mannheim kits (chem. 5 plus V2, Daman, India) on semi-automatic analyzer (Erba Mannheim). Experimental protocol for estimation of biochemical analysis was performed according to the methodology given in manual.

4.2.6.1 Estimation of creatinine
The concentration of creatinine in serum was determined by Erba Mannheim kit. Reagent 1 (500 µl picric acid) and Reagent 2 (500 µl sodium hydroxide) were mixed
and incubated at room temperature for 15 mins. Then, 50 µl of serum sample was added to the mixer and aspirated into the semi-automatic analyzer to get the concentration.

4.2.6.2 Estimation of urea
The quantitative estimation of urea was performed by Erba Mannheim kit. (Erba chem. 5 plus V2, Daman, India) Reagent 2 containing a mixture of ADP, gammalactate dehydrogenase (GLDH), urease, NADH, and α-ketoglutarate was dissolved in reagent 1 (phosphate buffer, pH 7.55). Serum sample (10 µl) was added to 500 µl reagent mixture and aspirated into the analyzer to get the concentration of urea.

4.2.6.3 Estimation of SGPT
The quantitative estimation of SGPT was performed by Erba Mannheim kit. Reagent1 500µl of L-alanine, NADH (yeast), lactate dehydrogenase (LDH), 2-oxoglutarate and tris buffer (pH 7.5) was mixed thoroughly with 500 µl of reagent 2. Serum sample (50 µl) was added to the mixture of reagent 1 & 2 and absorbance was noted at 340 nm.

4.2.6.4 Estimation of SGOT
The quantitative estimation of SGOT was performed by Erba Mannheim kit. Reagent1 500µl of 2-oxoglutarate, L-aspartate, malate dehydrogenase (MDH), lactate dehydrogenase (LDH), NADH (yeast), tris buffer (pH 7.8), and EDTA was mixed with reagent 2. Serum sample (50 µl) was added with mixture of reagent 1 and 2 and then absorbance was noted at 340 nm.

4.2.7 Preparation of tissue homogenate
Kidney tissue were excised and thoroughly washed in ice cold (0.1M) Phosphate buffer saline (PBS 1:9). Tissue was blotted on blotting paper and weighed to prepare 10% tissue homogenate in PBS and centrifuged at 10,000 xg for 20 mins at 4 °C. Supernatant obtained was used for enzyme assays, GSH and protein estimation.

4.2.7.1 GSH estimation
GSH in tissue and blood sample were determined according to the method of Beutler et al., (1963). This method is based on the development of a relatively stable yellow colour with DTNB with GSH.
Reagents preparation (Precipitating reagent)

For precipitating reagent 1.67 g metaphosphoric acid was added to 0.2 g EDTA and 30 g NaCl dissolved in 100 ml distilled water. Ellman’s reagent was prepared with 40 mg DTNB in 100 ml of 1% sodium citrate. For phosphate solution (0.3 M); 4.26 g of Na$_2$HPO$_4$ was dissolved in 100 ml of distilled water.

For blood sample

Blood sample (0.1 ml) was added with 0.9 ml of distilled water and 1.5 ml of precipitating agent. The samples was mixed gently and allowed to stand for 5 mins. The sample was centrifuge at 5000 xg for 5 min and 0.5 ml supernatant was added to 2 ml phosphate solution. Finally, 0.5 ml of Ellman’s reagent was added and optical density (O.D.) was measured at 412 nm immediately.

For Tissue sample

Tissue homogenate (1 ml) was added with 2 ml of precipitating solution. The homogenate was mixed and allowed to stand for 5 min at room temperature. The sample was centrifuged at 5000 xg for 5 min and 1 ml of supernatant was taken and mixed with 1.5 ml phosphate solution. Ellman"s reagent (0.5 ml) was added to it and optical density (O.D.) was measured at 412 nm immediately.

4.2.7.2 LPO assay

The method is based on the formation of a red chromophore that absorbs at 532 nm following the reaction of thiobarbituric acid (TBA) with malonyldialdehyde (MDA) and other break down products of peroxidized lipids collectively called as thiobarbituric acid reactive substances (TBARS) (Jamall and Smith, 1985).

To carry out LPO assay reaction mixture was prepared for positive control, blank, negative control and tissue homogenate. For positive control reaction mixture was prepared with 200 μl of 8% sodium lauryl sulphate (SLS) in 1.5 ml of 20% acetic acid and 1.5 ml of 1% TBA. Distilled water (800 μl) was added to the mixture to make the final volume up to 4.0 ml. Mixture was kept at 95 °C for 60 min in water bath followed by cooling immediately and centrifuged at 1000 xg for 5 mins at room temperature. The reaction mixture was acting as positive control and mixed with 2 ml of 10% TCA (Tricarboxylic acid) and O.D. was measured at 532 nm. For negative control, 1.5 ml of distilled water instead of 1.5 ml of 1% TBA was added to the reaction mixture. For blank 200 μl of 8% SLS was added with 1.5 ml of 20% acetic acid and
1.5 ml of distilled water was added to make the final volume up to 4.0 ml 800 μl of distilled water. Mixture was kept at 95 °C for 60 min in water bath. Tubes were cooled immediately under running tap water. Two ml solution was mixed with 2 ml of 10% TCA and O.D. was measured at 532 nm. For tissue analysis, 100 μl of tissue homogenate was added along with 1.5 ml of 1% TBA to make the final volume up to 4.0 ml by adding 700 μl of distilled water. Mixture was kept at 95 °C for 60 min in water bath. Tubes were cooled under running tap water immediately. Solution (2 ml) was added with 2 ml of 10% TCA and O.D was measured at 532 nm.

4.2.7.3 SOD Assay
The enzyme superoxide dismutase (SOD) dismutates superoxide to H₂O₂ and is therefore described as a superoxide quencher. The ability of the enzyme to inhibit the auto oxidation of pyrogallol in presence of EDTA was used as a measure of SOD activity (Marklund and Marklund, 1974).

Total 3 ml of reaction mixture was maintained throughout the experiment. Blank was prepared with 2.8 ml of 50 mM Tris-HCl buffer (pH 8.2) and 0.2 ml of 2 mM EDTA. In pyrogallol only 0.2 ml of EDTA was added to 2.6 ml of Tris-HCl buffer by addition of 0.2 ml of freshly prepared 0.2 mM pyrogallol. In case of tissue analysis 20 μl of 10% homogenate was added to 2.6 ml of Tris-HCl buffer and 0.2 ml of EDTA followed by addition of pyrogallol. Finally O.D. was taken at 420 nm with time intervals of 30 sec up to 3 minutes.

4.2.8 ¹H NMR spectroscopy of urine samples
Urine samples were centrifuged at 4722 xg for 5 min at 4 °C to remove particulate matter. Deuterated phosphate buffer solution (300 μl; 0.2 M Na₂HPO₄/0.2 M NaH₂PO₄, pH 7.4 containing 1 mM TSP prepared in D₂O) was mixed with 300 μl of centrifuged urine to minimize variations in pH of the urine samples. Urine sample mixed with deuterated phosphate buffer was then transferred to 5 mm NMR tube. TSP in phosphate buffer acted as chemical shift reference (0.0 ppm) and D₂O provided a signal lock. NMR spectral data were acquired on a Bruker-Av 400 spectrometer (Bruker, Rheinstetten, Karlsruhe Germany) with broadband observe (BBO) probe operating at a frequency of 400.13 Hz and temperature 298 K. Water signal was suppressed using water presaturation pulse; nuclear overhauser enhanced spectroscopy (NOESYPR1D) pulse
sequence (RD-90°-t-90°-tm-90°-acq) with relaxation delay (RD) of 2.0 sec, a mixing time of 50 millisecond and an acquisition time (acq) of 2.5 sec. Typically, 64 scans were collected into 32K data points over a spectral width of 6410 Hz.

4.2.9 1H NMR spectroscopy of serum

Serum sample (200 μl) was mixed with 400 μl of D2O and transferred to 5 mm NMR tubes containing 1 mM TSP closed capillary (Wilmad, SP Industries, Buena, NJ, USA) as reference. All NMR spectra were acquired at 400.13 MHz, Bruker-AV spectrometer (Bruker, Rheinstetten, Karlsruhe Germany) at 298 K. Water suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence (RD-90°-(τ-180°-τ)n-acquire) with a total spin echo (2nτ) of 200 millisecond was used to attenuate broad signals from proteins and lipoproteins. Here RD represents relaxation delay of 2 sec during which water resonance is selectively irradiated, 90° represents a 90° radio frequency (RF) pulse, τ is spin echo delay, 180° is 180° RF pulse and n represents number of loops. Typically 64 scans were collected into 32K data points over a spectral width of 8223.68 Hz with an acquisition time of 3.98 sec.

4.2.10 Perchloric acid extraction of polar metabolites in kidney

Polar metabolites were extracted from kidney tissue as described by Beckonert et al., (2007). Kidney tissue (100-120 mg) was homogenized with hand held homogenizer (IKA-T10, Germany) in 6 % ice-cold perchloric acid (5 ml/g of wet weight tissue). The homogenate was vortexed vigorously followed by incubation on ice for 10 mins. All samples were centrifuged at 12000 xg for 10 min at 4 °C. After centrifugation, the supernatant was neutralized to pH in physiological range (6.8-7.2) with drop by drop addition of 2 M KOH and left for 30 min on ice for precipitation of the potassium perchlorate salts. Precipitates were removed by centrifugation at 8000 xg for 10 min at 4 °C and the supernatant obtained was lyophilized and stored at -80 °C.

4.2.11 1H NMR spectroscopy of kidney tissue extracts

The lyophilized tissue extracts were reconstituted in 600 μl of D2O containing 1mM TSP and then vortexed vigorously followed with centrifugation at 4722 xg for 5 mins at 4 °C. The supernatant was transferred to 5 mm NMR tubes for 1H NMR spectroscopy. TSP acted as internal reference for NMR spectrum. 1H NMR spectra were acquired at 400.13 MHz on a Bruker Avance 400 spectrometer at 298 K using 1D ZGPR (RD-90°-
Acq) pulse sequence. For each sample, 64 transients were collected into 32K data points with a relaxation delay (RD) of 2 sec, flip angle of 90° and a mixing period of 100 millisecond. A spectral width of 9612 Hz and an acquisition time per scan of 2.56 sec was used.

The concentration of the metabolites was determined as mmole/g of wet tissue weight by comparing the integrated intensity of isolated resonance of the compounds of interest with that of the TSP signal, correcting for the number of contributing protons. The concentrations were calculated using the following formula:

\[ C = \frac{N_A \cdot A_A \cdot V_S}{B_N \cdot A_B \cdot W_S} \]

where; \( C_A \) is the concentration of the metabolite; \( C_B \) is the concentration of reference compound (mM/l), \( N_B \) is the number of protons in the reference compound; \( N_A \) is the number of protons in the peak of the metabolite; \( A_A \) is the intensity of the metabolite; \( A_B \) is the intensity of the reference compound; \( V_S \) is the volume of the sample (ml) and \( W_S \) is the wet weight of the sample (g).

4.2.12 NMR data processing and multivariate data analysis

NMR signals (FIDs) acquired during NMR spectroscopy from all the samples (serum, urine and tissue extract) were multiplied by an exponential weighting function corresponding to line broadening (0.3 Hz) before Fourier Transformation (FT). All the spectra of the study were phased and baseline-corrected manually using TOPSPIN 2.1 (Bruker, Germany). Each \(^1\)H NMR spectrum over the range 0.2–10.0 ppm was reduced to 245 regions of equal width (0.04 ppm) and the signal intensity in each region integrated using AMIX (Bruker, Germany). The region between 4.5 and 5.0 ppm was removed prior to any statistical analysis in order to eliminate any spurious effects of variability in the suppression of the water resonance. For urine spectra, the region containing urea (5.0–6.0 ppm) was also excluded to eliminate any crossrelaxation effects of urea. The urea peak is not quantitative as the protons of urea exchange with water and consequently the peak intensity varies with the quality of water suppression. Variation in urea peak intensity may highly influence NMR spectral data analysis. Following removal of these regions, data was normalized in AMIX by dividing each integrated segment by the total area of the spectrum to reduce any significant concentration difference. Output data in ASCII data format was imported to Microsoft Excel.
(Microsoft Office 2003), mean-centered and then exported to Matlab 7.1 (MathWorks, MA, USA) for pattern recognition (PR) analysis of NMR spectral data. Data were visualized by plotting the principal component (PC) scores, where each point on the score plot represents an individual sample. The resulting score values from PCA were subjected to one-way analysis of variance (ANOVA) to test the levels of significance between NiCl₂ treated rats and controls. In addition to the analysis carried out on the individual data, a metabolic trajectory was also constructed for the time course study. Metabolic trajectories derived from temporal studies can be useful in determining severity (magnitude) and recovery (return to normal) in response to a toxic insult. Prior to analysis, the segmented data for each experimental group at each time point were averaged resulting in a single data set/group thereafter PCA was carried out on these data to generate a metabolic trajectory. The predictive ability of combinations of biomarkers to phenotype NiCl₂ toxicity was applied to ¹H NMR spectra of urine samples exposed to the 3 doses over 120 h. Only nephrotoxic metabolite markers that had been fully characterised by NMR were used to ascertain whether NiCl₂ induced nephrotoxicity is responsible for the particular urinary metabolomic phenotype observed.

4.2.13 Statistical Analysis
The data obtained were expressed as Mean ± SD. The inter group variation was measured by one-way analysis of variance (ANOVA) followed by Fisher’s LSD multiple comparison procedure. Statistical significance was considered at P<0.05.

4.3 Results
¹H NMR spectroscopy based metabolic profiling of biofluids (serum and urine) and kidney and liver tissue extracts showed a number of metabolites from various metabolisms (Table 4.1). NMR based metabolic profiling revealed a number of metabolic alterations in biofluids as well as in kidney tissue extracts after NiCl₂ toxicity at different time points post dose. Multivariate data from ¹H NMR spectroscopy in conjunction of pattern recognition analysis displayed dose and time dependent response.

4.3.1 Histopathology and serum biochemistry
The histopathological findings (Fig. 4.1) after NiCl₂ administration caused prominent damage to the kidney compared to that of control (a). The kidney sections from NiCl₂ treated rats at 24 h p.d. showed acute damage to kidney tissues, which included
extravastion of erythrocytes in to the interstitium, tubular swelling, tubular degeneration and detachment of individual tubular cell from its basement membrane in both dose groups (b, c and d at 200X). At 120 h p.d., kidneys of rats treated with low dose of NiCl₂ (4 mg/kg b.w.) showed normal glomeruli and tubule-interstitial cells. In contrast, renal histopathological changes persisted up to 120 h p.d. in group of rats treated with high dose of NiCl₂ as evident from the tubular swelling and enlargement of glomeruli that were nonetheless observable in kidney sections.
Table 4.1: Metabolites from biofluids (urine, serum) and tissue extract (liver, kidney) and their role in different metabolism

<table>
<thead>
<tr>
<th>S No.</th>
<th>Metabolism</th>
<th>Urine Metabolites</th>
<th>Serum Metabolites</th>
<th>Liver Metabolites</th>
<th>Kidney Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Energy Metabolism</td>
<td>Citrate, (\alpha)-Keto glutarate, Fumarate, Succinate, Creatinine</td>
<td>Lactate, Alanine, Creatinine, Citrate, Succinate, (\alpha)-Ketoglutarate</td>
<td>Lactate, Alanine, Creatinine, Succinate (\alpha)-Ketoglutarate</td>
<td>Lactate, Alanine, Creatinine, Succinate (\alpha)-Ketoglutarate</td>
</tr>
<tr>
<td>2</td>
<td>Protein Metabolism</td>
<td>Branched Amino Acid, Glycine, Proline, Glutamine, Glutamate</td>
<td>Branched amino acid, Alanine, Glutamine, Glutamate</td>
<td>Branched amino acid, Glycine, Tryptophan, Histidine, Glutamine, Glutamate</td>
<td>Branched amino acid, Glycine, Sarcosine, Glutamine, Glutamate</td>
</tr>
<tr>
<td>3</td>
<td>Lipid Metabolism</td>
<td>Acetate, Acetoacetate, (\beta)-hydroxy butyrate (\beta)-LDL, VLDL, Unsaturated Fatty acid</td>
<td>Acetate</td>
<td>Acetate</td>
<td>Acetate</td>
</tr>
<tr>
<td>4</td>
<td>Gut Metabolism</td>
<td>Phenylalanine, Hippurate, TMA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Membrane Metabolism</td>
<td>Sarcosine, Choline, Phosphatidylcholine</td>
<td>Choline, Phosphatidylcholine</td>
<td>Choline, Phosphatidylcholine</td>
<td>Choline, Phosphatidylcholine</td>
</tr>
<tr>
<td>6</td>
<td>Nucleotide Metabolism</td>
<td>Nicotinate</td>
<td>-</td>
<td>Inosine, Nicotinuric acid</td>
<td>Inosine, Nicotinuric acid</td>
</tr>
<tr>
<td>7</td>
<td>Osmolytes</td>
<td>Myoinositol, Taurine</td>
<td>-</td>
<td>-</td>
<td>Myoinositol</td>
</tr>
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</table>
Fig. 4.1: Photomicrographs (200X) of kidney sections with haematoxylin-eosin under light microscope. (a) control rats showing normal kidney with normal tubular brush-borders (T) and intact glomeruli (G). No evidence of tubular inflammation or congestion was found in the tubules and glomerulus. (b) Rats injected with low dose of NiCl$_2$ (4 mg/kg) showing tubular swelling (TS), degeneration and stagnation (S) of RBC in the intestinal spaces, glomeruli and tubules at 24 h (c) and (d) Extensive damage to kidney tissue in terms of tubular swelling, stagnation of RBC and glomerular enlargement (GE) after administration of NiCl$_2$ (20 mg/kg) at 24 h.
Clinical parameters showed no significant change in the liver function in animals treated with NiCl\(_2\) compared to controls at all time points except for a decrease in AST at 10 mg/kg b.w. dose at 72 h and ALT at 24 h for the 20 mg/kg b.w. dose (Fig. 4.2 a and b). A significant elevation in serum urea and serum creatinine was observed at 24 h for all treatments. Thereafter, both continued to steadily decrease but did not return to p.d. concentrations (Fig. 4.2 d and e).

GSH was measured to determine antioxidant ability and was found to be significantly increased at 24 h p.d. in all animals treated with NiCl\(_2\) compared to controls (Fig. 4.2 c). The lower doses of 4 and 10 mg/kg b.w. showed an intermittent rise in GSH content as compared to the controls at 120 h p.d.

4.3.2 Antioxidant status of renal tissue from NiCl\(_2\) treated rats

To ascertain nickel induced oxidative stress in renal tissue, LPO, antioxidant enzyme such as SOD and reduced GSH content were measured (Table 4.2). NiCl\(_2\) induced oxidative stress in renal tissue was evident from increased LPO at both the dose groups at 24 h and 72 h p.d. At 120 h p. d., with low dose of NiCl\(_2\), LPO was comparable to control animals, whereas LPO was significantly increased in high dose treated group as compared to that of controls.

SOD activity was significantly increased in animals treated with high dose of NiCl\(_2\). The SOD activity in rats treated with high dose of NiCl\(_2\) continued to decrease with progression of time and at 120 h p.d. reached the same level as that of controls. After administration of NiCl\(_2\), there was significant increase in GSH content in both dose groups as compared to that of controls. Post treatment, the GSH content decreased with respect to time in both dose groups but was higher than that of controls.

4.3.3 \(^1\)H NMR spectroscopic analysis of serum

Assignment of metabolites

The representative \(^1\)D \(^1\)H NMR spectrums of serum shows a range of metabolites from lipid metabolism (LDL, VLDL, and acetate), amino acids (isoleucine, leucine, valine, alanine and glutamine/ glutamate) membrane metabolites (choline, phosphorethanolamine) osmolyte (betaine), and glucose (Fig. 4.3). These metabolites were assigned on the ppm scale of spectra viz. LDL, BAA, lactate, alanine, acetate,
**Fig. 4.2:** Changes in the level of functional parameters of liver, kidney and blood GSH in serum of rats treated with different doses of NiCl₂.

**Table 4.2:** LPO, activity of SOD and GSH content in kidney of rats treated with low and high doses of NiCl₂.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Parameters</th>
<th>Control</th>
<th>24 h</th>
<th>72 h</th>
<th>120 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>4 mg/kg b.w.</td>
<td>20 mg/kg b.w.</td>
<td>4 mg/kg b.w.</td>
<td>20 mg/kg b.w.</td>
</tr>
<tr>
<td>1.</td>
<td>LPO (nmoles MDA formed/hr/mg protein)</td>
<td>5.24±0.00</td>
<td>12.59±1.9*</td>
<td>7.75±0.36*</td>
<td>10.06±0.52*</td>
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<tr>
<td></td>
<td></td>
<td>8.47±1.36*</td>
<td></td>
<td></td>
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<tr>
<td>2.</td>
<td>SOD (units/mg protein)</td>
<td>7.18±0.36</td>
<td>7.24±0.42</td>
<td>11.06±1.3*</td>
<td>7.60±0.18</td>
</tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>3.</td>
<td>GSH (µmoles GSH/mg protein)</td>
<td>0.04±0.00</td>
<td>0.06±0.00*</td>
<td>0.09±0.00*</td>
<td>0.06±0.00*</td>
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</tbody>
</table>

Mean ± SD with *P < 0.05 at low and high dose of NiCl₂ treated group as compared with control.
Fig. 4.3: Representative 1D $^1$H NMR spectra of serum showing various metabolites in serum.
glutamine/glutamate, choline, phosphoethanolamine, betaine and glucose at 0.86, 0.94, 1.33, 1.48, 2.46, 3.21, 3.23, 3.25 and 3.54 respectively.

No significant change was observed in the $^1$H NMR spectra of serum after NiCl$_2$ except for the increase in the level lactate (Fig. 4.4)

4.3.4 Global urinary metabolomic changes in rat urine in response to NiCl$_2$ toxicity

A number of perturbations in endogenous metabolites were observed in the $^1$H NMR spectra of urine samples collected at different time points after NiCl$_2$ administration (Table 4.3). Spectral peaks of metabolites were assigned according to Lindon et al., (1999). Comparative urine $^1$H NMR spectra of different doses of NiCl$_2$ at 16 h and 120 h time points are presented in Fig. 4.5 (a) and 4.6 (a), respectively. Changes were observed in metabolites associated with energy metabolism (succinate, α-keto glutarate (α-KG), citrate, fumarate), gut flora metabolites (hippurate, trimethyl amine (TMA), dimethylamine (DMA)), osmolytes (trimethyl amine oxide (TMAO), taurine), membrane metabolites (choline and glycerophosphocholine (GPC)), amino acids (branched chain amino acids (BAA), phenylalanine), N-acetylglutamate (NAG), N-methylnicotinamide and creatinine. After the first 8 h p.d. a decrease in NAG, acetoacetate, fumarate, hippurate, N-methylnicotinamide and formate levels was observed at all three doses. In addition, TMAO and choline levels decreased at the higher doses (10 and 20 mg/kg b.w.) whilst succinate and α-KG levels decreased only at the low dose (4 mg/kg b.w.) as compared to controls. Contrary to these changes, at 16 h p.d. energy metabolite levels were increased in animals treated with a low dose of NiCl$_2$ as compared to controls. In the high dose treatment the changes that were observed during first 8 h became more prominent by 16 h. At 24 h p.d., the low dose group showed an increase in the majority of metabolites whereas, most of the metabolite levels were still reduced at the higher dose of NiCl$_2$. After 72 h, most of the metabolites in the low dose group returned to normal levels as compared to controls, however, changes were still persistent in citrate, creatinine, choline, TMAO, GPC, fumarate and phenylalanine levels in animals treated with the higher dose of NiCl$_2$. 
Fig. 4.4: Representative $^1$H NMR spectra of serum from rat exposed to three doses of (4, 10 and 20 mg/kg b.w.) NiCl$_2$ at 24 h.

Table 4.3: Summary of NMR spectroscopy based NiCl$_2$ induced variations in urinary metabolites induced by 3 doses of NiCl$_2$ as compared to controls.
<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Characteristic chemical shifts (delta, multiplicity)</th>
<th>Time point of observed decrease in metabolite signal intensity in h</th>
<th>Time point of observed increase in metabolite signal intensity in h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 mg/kg b.w</td>
<td>10 mg/kg b.w</td>
</tr>
<tr>
<td>BAA</td>
<td>0.9-1.05 (m)</td>
<td>-</td>
<td>72-96</td>
</tr>
<tr>
<td>NAG</td>
<td>1.89, 2.06 &amp; 2.1 (m)</td>
<td>8</td>
<td>0-96</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.43 (s)</td>
<td>8</td>
<td>16-96</td>
</tr>
<tr>
<td>α – K.G.</td>
<td>2.45 (t), 3.01 (t)</td>
<td>8</td>
<td>16-96</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.55 &amp; 2.67 (AB)</td>
<td>24</td>
<td>16-120</td>
</tr>
<tr>
<td>DMA</td>
<td>2.72 (s)</td>
<td>-</td>
<td>16-96</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.05 (s), 4.06 (s)</td>
<td>-</td>
<td>16-120</td>
</tr>
<tr>
<td>Choline</td>
<td>3.21 (s), 3.5 &amp; 4.07 (m)</td>
<td>-</td>
<td>0-120</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.27 (s)</td>
<td>-</td>
<td>16-120</td>
</tr>
<tr>
<td>GPC</td>
<td>3.35 (s), 3.52 (m)</td>
<td>-</td>
<td>120</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>3.45 (s)</td>
<td>8, 24</td>
<td>8, 72</td>
</tr>
<tr>
<td>Fumarate</td>
<td>6.53 (s)</td>
<td>8, 72-120</td>
<td>0-120</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.33 (m), 7.38 (m)</td>
<td>72-120</td>
<td>16-120</td>
</tr>
<tr>
<td>Hippurate</td>
<td>7.55 (t), 7.64 (t), 7.84 (d)</td>
<td>8</td>
<td>0-24, 96</td>
</tr>
<tr>
<td>NMN</td>
<td>4.48 (s), 8.95, 8.97, 8.9 (d), 8.19 (t)</td>
<td>0-16, 72</td>
<td>0-96</td>
</tr>
<tr>
<td>Formate</td>
<td>8.46 (s)</td>
<td>0-16, 72-120</td>
<td>0-96</td>
</tr>
</tbody>
</table>

Keys: s: singlet; d: doublet; t: triplet; m: multiplet. P < 0.05 for all the changing metabolites as compared to controls.
Representative spectra for control and treated animals has been shown in fig. 4.5 a and 4.6 a
Fig. 4.5: Representative $^1$H NMR spectra of urine and PCA plots from rat exposed to three doses of (4, 10 and 20 mg/kg b.w.) NiCl$_2$ at (a, b) 16 h
Fig. 4.6: Representative $^1$H NMR spectra of urine and PCA plots from rat exposed to three doses of (4, 10 and 20 mg/kg b.w.) NiCl$_2$ at (a, b) 120 h
Throughout the study, perturbation could be clearly seen in most of the metabolites but most prominent changes were observed in citrate, DMA, creatinine, choline, TMAO, phenylalanine and hippurate. Fig. 4.7 shows changes in the relative intensity levels of these metabolites as the time progressed in all three dose groups.

4.3.5 Pattern recognition (PR) analysis of urine sample
Urine samples collected at all doses and time points of the study were assessed using PCA analysis to investigate both dose and time dependent effect of NiCl$_2$. PCA proved to be a useful and rapid means of establishing whether the urine spectra obtained from rats treated with NiCl$_2$ were different from those obtained from control animals and also of identifying at which time points maximum biochemical effect occurred. Significant difference (ANOVA, P < 0.05) on PC1 and PC2 scores were observed between NiCl$_2$ exposed groups and the controls at the earlier time points (0-72 h p.d). At 0-8 h p.d. overlapping of score values of NiCl$_2$ treated groups (4 and 10 mg/kg b.w.) with pre-dose values were observed. At 16 (Fig. 4.5 b), 24 and 72 h p.d. time points, all three NiCl$_2$ treatments were clearly separated from controls. However, by 96 h the scores plot showed a clear separation of control from the high dose (20 mg/kg b.w.) group only and partial overlapping was observed between control and 4 and 10 mg/kg b.w. dose groups (Fig. 4.8). Analysis of scores plot at 120 h p.d. (Fig. 4.6 b) illustrated segregation of high dose groups (10 and 20 mg/kg b.w.) from control and low dose (4 mg/kg b.w.) group. The metabolic trajectory shown in Fig. 4.9 clearly showed the progression of NiCl$_2$ induced toxicity leading to maximum change in metabolite levels by 24 h. In case of low dose group, after 24 h the trajectory started to return to the control levels as the urinary metabolic profile gradually recovered despite some differences in the levels of a few metabolites whereas, for the high dose groups, the trajectory returned back to normal levels only after 96 h but still did not realign with control values confirming that changes in metabolic profile still persisted. Furthermore, animals treated with 10 mg/kg b.w. of NiCl$_2$ showed proximity and deviation from control at later time points.
Fig. 4.7: Relative changes in levels of urinary (a) citrate, (b) creatinine, (c) TMAO and (d) hippurate, measured by $^1$H NMR, as a function of time and NiCl$_2$ dose. Data presented as relative levels with respect to total spectral area ± standard deviation, with overall differences between all groups ($P = 0.05$) and post hoc tests ($P = 0.05$) revealing a difference from control levels.
Fig. 4.8: PCA Scores plot of $^1$H NMR spectra of urine sampled at 96 h from control rats (○, n=5) and NiCl$_2$ doses with 4 (●, n=5); 10 (■, n=5); and 20 (▲, n=5) mg/kg body weight dose.
**Fig. 4.9:** PCA time trajectory plot mapping the average position of $^1$H NMR spectra from (a) 4 mg/kg body weight, (b) 20 mg/kg body weight and (c) 10 mg/kg body weight NiCl$_2$ treated rats for each time interval.
4.3.6 Urinary metabolomic phenotyping for detection of NiCl₂ induced nephrotoxicity

The combination of pairs of nephrotoxic metabolite biomarkers identified in urinary spectral profiling, specifically creatinine and citrate, creatinine and TMAO together with citrate and TMAO were evaluated for their ability to define a urinary metabolomic profile that could diagnose NiCl₂ induced nephrotoxicity. At 8 h p.d., only the high dose group segregated from control with respect to all three biomarkers combinations. However, at 16 h and 24 h p.d. plots of creatinine as a function of citrate and TMAO as a function of citrate segregated into different clusters (Fig. 4.10). The plot of TMAO as a function of creatinine was not informative for the low dose group. After 72 h p.d. there was no discernible difference between the low dose treatment and the control, however, there was segregation between these and the higher dose treatments for creatinine as a function of citrate and TMAO as a function of citrate. There was no segregation observed in the plot of TMAO as a function of creatinine.

4.3.7 ¹H NMR spectroscopic analysis of kidney tissue extract from NiCl₂ treated rats

Representative ¹H NMR spectra of kidney polar extracts are shown in (Fig. 4.11 a and 4.11 b). Spectral peaks of metabolites were assigned according to Lindon et al., (1999). The resonance assignment of various metabolites was carried out on 1D ZGPR spectrum of tissue extract. Thirteen major metabolites were unambiguously assigned, which included amino acids, organic acids (citric acid cycle intermediate and product) and other important metabolites (Table 4.4).

The first important metabolite in tissue extract was a group of branched amino acids (isoleucine, leucine, valine) and the peaks appeared between 0.8–1.04 ppm due to methyl proton in 1D NMR spectrum. The doublet and quartet resonance structure for β-CH₂ protons and α-CH protons of alanine were identified at 1.47 and 3.77 ppm, respectively. The two non equivalents β-CH₂ protons of glutamate are coupled with α and γ–CH₂ protons giving rise to a complex multiplet resonance in the 1D spectrum. The β-CH₂ protons of glutamine were equivalent and nearly overlap with β-CH₂ protons of glutamate in the 1D NMR spectrums in between 1.95 – 2.15 ppm. The β-CH₃ protons of lactate at 1.33 ppm showed sharp doublet peak and at 4.12 ppm quartet
**Fig. 4.10:** Predictive ability of biomarker combinations for NiCl$_2$ exposure in rats. Panel I: control compared to all the three doses at 16 h (a) creatinine and citrate; (b) TMAO and citrate; (c) TMAO and creatinine. Panel II: control compared to all the three doses at 72 h. (d) creatinine and citrate; (e) TMAO and citrate; (f) TMAO and creatinine.
Fig. 4.11 a: Representative $^1$H NMR kidney tissue spectra from control rat and exposed to low and high doses of NiCl$_2$ at 24 h post dose.
Fig. 4.11 b: Representative $^1$H NMR kidney tissue spectra from control rat and exposed to low and high doses of NiCl$_2$ at 120 h post dose.
Table 4.4: Summary of NMR spectroscopy based NiCl$_2$ induced variations in kidney tissue as compared to controls

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift with multiplicity (Bracket)</th>
<th>24 h</th>
<th>72 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 mg/kg b.w.</td>
<td>20 mg/kg b.w.</td>
<td>4 mg/kg b.w.</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d), 4.12 (q)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d), 3.79 (q)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>2.10, 2.14, 2.46, 2.50 (m), &amp; 3.77 (t)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.36 (m)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.43 (s)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.04 (s)</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Choline</td>
<td>3.21 (s), 3.52 (m), 4.07 (m)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>PEA</td>
<td>3.23 (t), 4.00 (m)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.27 (s)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.25 (t), 3.43 (t)</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.57 (s)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>2.74 (s), 3.61 (s)</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>3.28 (t), 3.56 (dd), 3.63 (dd)</td>
<td>↑</td>
<td>↓</td>
<td>-</td>
</tr>
</tbody>
</table>

↑, indicates increase in concentration of selected metabolite; selected metabolite. Keys: s: singlet; d: doublet; t: triplet; m: multiplet. P < 0.05—, no change ; ↓, indicates relative decrease in concentration of for all the changing metabolites.
due to α-CH protons. Singlet due to methyl (α-CH\textsubscript{3}) group of acetate and β-CH\textsubscript{2} group of succinate were observed at 1.91 and 2.40 ppm respectively. The +N(CH\textsubscript{3})\textsubscript{3} groups of choline and phosphoethanolamine (PEtha) appeared in narrow chemical shift range of 3.20-3.24 ppm. The singlet observed at 3.20 due to +N(CH\textsubscript{3})\textsubscript{3} moiety of choline and at 3.22 ppm due to GPC. However it’s difficult to distinguish between phosphoethanolamine and GPC in 1D NMR spectra.

Due to the removal of cellular lipid components, the majority of peaks in the spectra are well resolved to make metabolite assignments relatively direct. Major endogenous metabolites for kidney homogenates contained products of glycolysis (glucose, lactate), amino acids, organic osmolytes [e.g., betaine, myo-inositol, taurine and trimethylamine N-oxide (TMAO)], membrane metabolites (choline, phosphorylethanolamine) and creatine. \textsuperscript{1}H NMR spectroscopic analysis of kidney tissue extracts from NiCl\textsubscript{2} treated rats revealed a variety of biochemical changes. \textsuperscript{1}H NMR spectral profile of kidney tissue extract from NiCl\textsubscript{2} treated animals showed dose as well as time dependent changes (Table 4.4). At 24 h p.d., rats treated with high dose of NiCl\textsubscript{2} (20 mg/kg b.w.) showed significant decrease in the concentration of all metabolites, whereas, rats exposed to low dose of NiCl\textsubscript{2} (4 mg/kg b.w.) showed significant increase in all the metabolites except creatine and sarcosine. Fig. 4.12 shows changes in metabolites concentration at different post dose time point. With the progression of time, animals treated with lower dose of NiCl\textsubscript{2} (4 mg/kg b.w.) showed signs of recovery as there was no significant difference in the concentrations of most of metabolites observed in NMR spectra at 72 h and 120 h p.d. For rats treated with 20 mg/kg b.w. dose of NiCl\textsubscript{2}, changes in the NMR spectral profile continued till 72 h and 120 h p.d. and concentration of most of the metabolites was significantly reduced compared to that of the controls.

\textbf{4.4 Discussion}  
Toxicological studies are mainly based on histopathological evaluation and on serum or plasma biochemical examination. The NMR-based metabolomic approach is a technique granting researchers a variety of low molecular-weight metabolites that could be used as biomarkers for a specific pathological state or a study of toxic insult.
Fig. 4.12 Relative changes in the concentration of selected metabolites in the kidney tissue spectra at different time points of control (■) low (○) and high (●) dose groups of NiCl$_2$ treatment. Data presented as relative levels with respect to total spectral area ± standard deviation, with overall differences between low (○) and high (●) dose groups (P < 0.05) and post hoc tests (P < 0.05) revealing a difference from control (■) level.
High resolution $^1$H NMR spectroscopic analysis of biofluids has proved to be one of the most powerful techniques for investigating the response of organisms to xenobiotics. The aim of the current study is to investigate metabolic consequences of NiCl$_2$ exposure in a holistic manner by employing a metabolomic strategy. The above results indicated that NiCl$_2$ exposure caused significant renal toxicity and comprehensive metabolic changes in urine involving many related metabolic pathways.

During the first 16 h p.d. a marked change in the urinary metabolic profile (as detected by $^1$H NMR spectroscopy) was observed with a notable reduction in the levels of the tricarboxylic acid (TCA) cycle intermediates such as citrate, succinate and $\alpha$-KG. Depletion of urinary citrate, a normal component of rat urine has been attributed to either an impairment of the TCA cycle or renal tubular acidosis (Hauet et al., 2000). Moreover, the observed decrease in these energy metabolites with concomitant increase in serum lactate (Fig. 4.4) might be indicative of enhanced anaerobic metabolism. Our observations suggested that energy metabolism was perturbed at an early stage in the development of NiCl$_2$ induced injury. The $^1$H NMR metabolic profile observed following administration of NiCl$_2$ was similar to that seen following administration of HgCl$_2$ or other heavy metals, which induced damage to the proximal tubule (Holmes et al., 1998; Wang et al., 2006; Wu et al., 2006; Peiqiu et al., 2009). The formation of free radicals in various tissues, which leads to modifications of DNA bases, enhanced LPO and altered calcium and -SH homeostatis has been suggested as the primary cause for nickel toxicity (Das et al., 2008). It has been observed that, under oxidative stress, the mitochondrial TCA cycle is slowed down in cellular regulation to reduce the natural production of free radicals (Singh, 2006). The reduction in the level of BAA 72 h p.d. onwards further supports impairment of energy metabolism, as these could be consumed as energy precursors in order to sustain energy demand during halt of Kreb cycle.

In the present study, decreased TMAO level was observed in the dosed rats as time progressed suggesting that NiCl$_2$ exposure initiated damage to medullary region. TMAO is one of the major medullary osmolytes required for maintaining renal osmolyte homeostasis (Garrod et al., 2001) therefore; it may be possible that decreased TMAO occur as a result of osmotic dysregulation in renal tissue due to NiCl$_2$ induced toxicity. Furthermore, decreased levels of hippurate, phenylalanine and other aromatic compounds were observed in urine from animals treated with the higher dose of NiCl$_2$. This implies that NiCl$_2$ exposure may result in poor intake or improper digestion of food as the urinary levels of hippurate and other aromatic compounds have been shown to correlate with dietary (Phipps et al., 1998) and intestinal...
microbiota changes (Gavaghan et al., 2001; Nicholls et al., 2003; Roboscoky et al., 2005). Previous finding has supported the notion that exposure to nickel induced changes in the expression of intestinal enzymes (Singla et al., 2006). NiCl₂ induced changes in urinary hippurate and other aromatic amino acids and compounds were not only reflective of dietary intake, but variations of these urinary aromatic compounds are frequently related to modulation of activity or populations of gut microbiota. Therefore, NiCl₂ exposure may have caused changes in gut microbiota, which necessitates further investigation to address the challenges to microbiome.

Rats administered with NiCl₂ exhibited significantly reduced levels of choline and N-methyl nicotinamide in urinary NMR spectral profiles of high dose group animals (10 and 20 mg/kg b.w.). Such depletion of these metabolites could be as a result of the antioxidant response triggered by NiCl₂ induced oxidative stress. Choline and N-methyl nicotinamide are a source of methyl during regeneration of S-adenosylmethionine from homocysteine. The S-adenosylmethionine thus formed may be utilized in the transsulfuration pathway to regenerate GSH stores, which are depleted in order to detoxify the reactive oxygen species (ROS) generated during nickel exposure (Sun et al., 2008). This is further supported by the observed elevated GSH levels in blood during nickel toxicity.

The changes in clinical chemistry parameters were consistent with renal injury and demonstrated the progression of and recovery from NiCl₂ induced nephrotoxicity. The reduction of creatinine in urinary metabolic profiles was consistent with the elevated levels of creatinine in the serum. The elevation of urea and creatinine in blood at 24 h could be a result of renal injury during nickel toxicity, which is also supported by histopathological findings as well as kidney tissue extract spectroscopy. The increase in serum urea was observed to be higher as compared to creatinine at 72 and 120 h, probably due to lower clearance of urea as compared to creatinine. Similar kind of changes in serum creatinine and blood urea nitrogen were observed upon exposure to different toxic substances (Wu et al., 2005; Wei et al., 2008). In addition, a decrease of creatinine in urine as shown by NMR studies is related to the lower rates of glomerular filtration due to perturbation of the rennin–angiotensin system (Feng et al., 2002).

PCA of urine ¹H NMR spectra demonstrated the dose and time dependent development of toxicity. The ability to distinguish between low and high dose data sets suggested that metabolic perturbations that characterize NiCl₂ induced nephrotoxicity varied with increasing dose. This was demonstrated by relative quantitation of urinary citrate, creatinine, TMAO and hippurate, which was excreted in a dose dependent manner (Fig. 4.7). The sporadic increase in
the levels of creatinine, TMAO and hippurate in urine at 72 h p.d. especially in the case of the higher dose groups (10 and 20 mg/kg b.w.) might be dose specific changes. After 72 h the 4 mg/kg b.w. group recovered, while the changes still persisted in high dose group till 120 h. The onset of the recovery process was also evident as the metabolic trajectory turned back towards control levels after 24 h p.d. in case of low dose group. For the higher dose groups, the p.d. levels moved towards the control level from 96 h onwards, however the return to control values by the end of the monitoring period was not observed. However, the erratic behaviour of 10 mg/kg b.w. dose group at later time points (96 and 120 h) may be because of an unpredictable counter response of the body against NiCl₂ toxicity. Furthermore, the changes detected in urine spectral profiles with special reference to renal metabolism viz creatinine, energy metabolites and osmolytes were consistent with the changes observed in NMR spectra of renal tissue extract after NiCl₂ intoxication. The present study on urine NMR spectral profiling not only showed NiCl₂ induced nephrotoxicity but also other pathophysiological changes related to oxidative stress, nucleotide and amino acid metabolism. NMR spectra of biofluids such as urine have been considered as a helpful tool either for quantitative metabolic-fingerprinting or as a means of determining metabolite (biomarker) structure (Nicolson et al., 2002). It was not possible to identify unambiguously all urinary constituents that were elevated after NiCl₂ exposure; even so, information collected on these constituents might still be useful as additional biomarkers of NiCl₂ toxicity. The pairing of metabolic biomarkers (Fig. 4.10) might ultimately improve predictions of nickel induced renal toxicity. In addition, these metabolic biomarker pairings would help us in predicting the correlation between the known nephrotoxic biomarkers and further how these metabolites were interdependent on each other with respect to dose and time. The results of ¹H NMR spectroscopy of renal tissue extract demonstrated metabolic perturbations in some of the important physiological systems of kidney during acute toxicity of NiCl₂. Prominent changes observed in NMR spectra of kidney tissue extracts were mainly associated with energy metabolism, osmolytes, amino acids, glucose and membrane metabolism.

In the present study, strikingly different physiological/metabolic response in renal tissue in terms of NMR spectral profiles was observed. There was an increase in most of the metabolites at 24 h in animals exposed to low dose of nickel, whereas, rats treated with high dose of nickel revealed a continuous decrease in all the metabolites. The histopathological findings were more pronounced in animals exposed to high dose as compared to those exposed to low dose of NiCl₂. In animals treated with high dose of NiCl₂, NMR spectral profile
alterations were substantiated very well with histopathological changes in terms of tubular swelling and glomerular enlargement. Similar observations have also been detected in other heavy metal toxicity studies (Huifeng et al., 2006; Adewole et al., 2007). The reduced metabolites concentration in high dose group compared to that of controls and low dose group clearly suggested acute renal injury due to nickel toxicity. Acute tubular injury, which is generally reversible and seen in association with drug or heavy metal toxicity in terms of tubular swelling, not only affects kidney function but may also lead to renal tubular dysfunction with or without renal failure. Kidney is a dynamic organ, rich in enzymes required for amino acid catabolism and/or oxidation, in particular in the medullary thick ascending limb, which provides energy to sustain active ion transport (Guder and Ross, 1984). The decrease in amino acids at high dose group of NiCl₂ treated group at 24 h p.d. may be associated with severe damage to the tubules, as 99% of filtered amino acids are reabsorbed in the proximal tubule. Increased amino acid excretion in urine due to impaired reabsorption by the renal tubules and increased cellular permeability of the glomerular membranes during nephrotoxic conditions has been reported by Macpherson et al., (1991). Several earlier NMR spectroscopy based studies in rare earth or heavy metal induced toxicity showed increased branched amino acid, succinate, creatinine and DMA level in urine (Feng et al., 2002; Huifeng et al., 2006). Nickel affects membrane properties and oxidation/reduction system intracellularly due to a high affinity for cellular structure or ion channels (Refvik and Andreassen, 1995). The reduction in osmolytes (myo-inositol, betaine, TMAO) in the present study showed disturbed renal homeostasis. Organic osmolytes such as betaine, sorbitol and myo-inositol are present in especially high concentrations in the inner medulla (Niemann and Serkova, 2007). Reduced levels of osmolytes can very well be associated with degenerative changes observed histologically in the medullary regions. Likewise reduced energy metabolites (lactate, succinate, creatine) in NMR spectra of renal tissue indicate cortical as well as medullary damage. In case of animals exposed to high dose of NiCl₂, the results are in agreement with earlier toxicity based studies that reported decrease in the level of renal osmolytes (GPC, myo-inositol and betaine) in intact renal tissue or in urine (Garrod et al., 2001; Wang et al., 2006).

The animals exposed to low dose of NiCl₂ exhibited increase in most of the metabolites at 24 h p.d. Some histological damage was reflected in these animals but they recovered by 72 h and 120 h p.d. It implied that changes observed during 24 h p.d. may not be due to tubular injury but due to some other indirect nickel toxicity. The increased metabolite levels might be due to an immediate stress induced response of the body towards toxic insult (Tyagi et al., 2011). There are several
NMR spectroscopy based heavy metal induced toxicity studies that have shown increased anaerobic metabolism and decreased energy metabolites as oxidative stress sets in (Feng et al., 2002; Lenz et al., 2004; Tyagi et al., 2011). In the present study, increased lactate and arresting of tricarboxylic acid (TCA) at succinate level (as ascertained by increased succinate) exhibit oxidative stress induced changes in energy metabolism. The elevated levels of glutamate in renal cortex samples from NiCl₂ (4 mg/kg b.w.) treated rats may arise from the inhibition of malate and succinate dehydrogenases causing a build up of α KG in the TCA cycle (Curthoys and Watford, 1995). The renal metabolism of glutamine, the major precursor of the ammonium ions excreted in urine, is of central importance for the regulation of systemic acid-base homoeostasis and for energy provision to renal cells. Increased oxidative stress may also lead to disturbance of cellular integrity, which may further lead to activation of degradable enzymes resulting in cytotoxicity (Catala, 2009). Membrane phospholipids viz phosphorylethanolamine has been evaluated as a biomarker of cytotoxicity (Hwang and Kim, 2007). Increased level of phosphorylethanolamine and choline in low dose group animal in the present study was in agreement with oxidative stress induced cytotoxicity.

Depletion of both medullar (TMAO) as well as papillary osmolytes suggested massive damage to all kidney tissue due to NiCl₂ toxicity in high dose group animals, whereas, in case of low dose group, increase in level of renal osmolytes such as taurine, myo-inositol and amino acids such as alanine, glycine, and glutamine might be due to NiCl₂ induced osmotic stress. Apparently, oxidative stress may lead to decreased medullary blood flow, Na⁺ excretion (hypertension) and osmotic stress, resulting in the release of medullary osmolytes. It has been shown in an earlier study that in response to hypertonic stress, there was an accumulation of myo-inositol and amino acids in the renal medulla (Bagnasco et al., 1986). In another study on renal injury, amino acids were suggested as renal osmolytes against injury and involved in a process critical for maintaining cellular integrity (Weinberg et al., 1990).

Animals exposed to low dose of nickel showed oxidative stress as evident by the increase in renal LPO. Reduced LPO and increased GSH content at low dose of nickel indicate the efficiency of the antioxidant system in counteracting NiCl₂ induced toxicity. However, SOD activity was not elicited at low dose of nickel exposure. The earlier studies based on nickel induced toxicity have shown higher αOH levels compared to those of control, while catalase, mannitol and GSH provided protection against nickel induced oxidative stress (M"Bemba-Meka et al., 2005).
Another study showed increased LPO in kidney tissue and demonstrated reverse correlation of nickel induced LPO with the extent of its effect on glutathione, glutathione peroxidase (GPx), glutathione reductase (GSH), but not with catalase, SOD or glutathione -S- transferase (GST) (Misra et al., 1990). However, animals exposed to low dose of NiCl₂ showed lowered LPO and were able to cope up with increased oxidative stress as shown by sustained level of GSH up to 120 h p.d, whereas high dose nickel resulted in increased LPO with significant decrease in GSH even at 120 h after exposure.

In animals treated with lower dose of NiCl₂, most of the metabolites in NMR spectra of kidney tissues as well as antioxidant enzyme assays has reached to normal level by 72 h onwards and were comparable to those of controls. This may be due to the fact that the pharmacokinetics of the nickel is very rapid and has low retention time in the body with a half life of 20-60 hrs (Rezuke et al., 1987). The animals receiving high dose of NiCl₂ showed decreased metabolite levels even at 72 h and 120 h p.d. indicating the inability of the body to cope with nickel induced acute renal injury.

4.5 Conclusion

The present study established the utility of metabolomic approach based on ¹H NMR spectroscopy of urine as well as the renal tissue extracts that demonstrated NiCl₂ induced renal damage in rat. A differential response was observed in renal tissue, when exposed to low and high dose of nickel, in terms of metabolites as observed by NMR based metabonomics. The time and dose dependent changes in endogenous metabolite in kidney extracts could illustrate obvious biochemical pathways perturbed by NiCl₂ administration. Correlation of ¹H NMR spectroscopy analysis with histology and antioxidant enzyme assay further confirms the reproducibility of the NMR technique. The ¹H NMR spectroscopy study of urine took the advantages of available authentic references and successfully identified 16 endogenous components in rat urine related to metabolic response after NiCl₂ exposure. Identification of these metabolic markers has enabled us to elucidate the pathological status as well as the underlying relationships between clinical biochemical fluctuations and metabolic disequilibria caused by toxic stimulation. In addition, identification of metabolic phenotypes based on pairs of biomarkers may ultimately be helpful in predicting nickel induced nephrotoxicity. Therefore, information from metabolomic approach may lead to more comprehensive biochemical characterization of the perturbations that occur in various toxicity pathways.