CHAPTER-6

NMR BASED METABOLOMICS

AFTER Na$_2$WO$_4$.2H$_2$O INDUCED TOXICITY
6.1 Introduction

Tungsten is one of the rare metals comprising about 1.5 ppm of earth crust. It is a complex metal that is widely used in commercial and military applications due to its tensile strength and high melting point (Koutsospyros et al., 2006). Elemental tungsten is basically insoluble and as a result is considered to be of low toxicity and it has not been implicated in health and risk assessment issues in humans and the environment. Soluble compounds are more toxic than insoluble form. Besides occupational hazard, tungsten compounds pose environment hazard and human can be exposed to tungsten and its compound released in environment through its widespread usage in steel, manufacture of alloys, light filaments of X ray tubes and bulbs, fire and water proofing fibres. A small amount of tungsten is also released through its military usage as heavy metal tungsten alloy in ammunitions. Few toxicological studies have been conducted on tungsten compounds and these compounds have been known to cause more acute health effects than chronic health effects. However, the toxicological effects of tungsten are still poorly understood as compared to other heavy metals.

Tungsten has been shown to act by antagonising the action of the essential trace element Molybdenum (Mo). Due to presence of high concentration of molybdenum dependent enzymes in liver, liver has become one of the target organs for tungsten induced toxicity (Johnson et al., 1974 a and b). In addition, tungstate polymerising with phosphate readily make phosphotungstate thereby interacting with several phosphate dependent biochemical pathways in the body. Recently, Johnson et al., (2010) have shown negative effect of tungsten phosphate on several intracellular phosphate dependent pathways in cell lines suggesting that tungsten may affect cellular biochemistry. It is anticipated that tungsten exposure may indirectly result in disturbed homeostasis and its exposure may lead to altered physiology or intermediary metabolism of liver, although no such report have been published so far. With the traditional biochemical method focussing on a single metabolic pathway at a time, a holistic systemic response remains to be investigated to further the understanding of metabolic effects of tungsten induced toxicity. Metabolomic technology has proven itself one of the suitable tool for studying systems metabolic responses to many toxins and related metabolism (Waters et al., 2001; Wojnowski et al., 2004; Yap et al., 2006; Ebbels et al., 2007).

Metabonomics is a multi-parametric approach that allows detection of the metabolic response due to chemical exposure (Feng et al., 2002; Wei et al., 2008) and has
previously been used for environmental (Viant et al., 2003), pharmaceutical (Coen et al., 2004), biomarker discovery (Jordan and Cheng, 2007) and toxicology (Shockcor and Holmes, 2002) investigations. Metabolomics involves an analysis of metabolic profiles of biofluids or tissue extracts using $^1$H NMR spectroscopy or mass spectrometry in conjunction with multivariate data analysis (Azmi et al., 2005). NMR spectroscopy based metabolomics have been widely used for analysis of biofluids and tissues since it is non-destructive, highly reproducible, nonselective to detectable metabolites, provides structural information and quantative. Several metabolomics studies have been employed to study the organ-specific biochemical responses of the body to number of toxins (Coen et al., 2003; Garrod et al., 2005; Ding et al., 2009). The effectiveness of metabolomic data to determine the effects of hepatotoxic and nephrotoxic compounds using NMR spectroscopy of urine and tissue extract have been reported (Kleno et al., 2004; Portilla et al., 2006; Beger et al., 2010; Lu et al., 2010). The information regarding the toxic effect of tungsten at metabolite level is inadequate. Therefore in the present study, analysis of tungsten induced metabonomic changes in rat urine, serum, liver and kidney tissue extract using NMR-based metabolomic approach coupled with clinical chemistry and histopathology of liver and kidney tissues has been studied. The objectives of this study are to investigate the multiple-matrix metabolic responses of rats to tungsten exposure and to further the understanding of tungsten toxicity at the systems level.

6.2 Materials and methods
All chemicals, NMR solvents, trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP) and deuterium oxide (D$_2$O), Na$_2$WO$_4$. 2H$_2$O, used in the study were obtained from Sigma-Aldrich (St. Louis, MO, USA). All animal handling and experimental protocols conformed to the guidelines stipulated by the Institutional Animal Ethical Committee.

6.2.1 Animal handling and sample collection
A total of 60 male Sprague Dawley (SD) rats, 11 weeks of age (250±20 g) were obtained from experimental animal facility of the institute and housed in group of five in polypropylene cages at a controlled temperature of 22±2 °C and a relative humidity of 50±10%. Food and tap water were provided ad libitum. The light cycle was maintained at 12 h of light and 12 h of darkness. After acclimatisation for a week, rats
were randomly divided into three groups (n = 18 in each group) and subjected to sodium tungstate (Na$_2$WO$_4$. 2H$_2$O) treatment. Na$_2$WO$_4$. 2H$_2$O was prepared in saline and injected intraperitonealy (i.p.) at a dose rate of 40.8 (low dose, one fifth of LD$_{50}$ of Na$_2$WO$_4$. 2H$_2$O), 122.4 (moderate dose, three fifth of LD$_{50}$ of Na$_2$WO$_4$. 2H$_2$O) and 204 mg/kg body weight (b.w.), (high dose, LD$_{50}$ of Na$_2$WO$_4$. 2H$_2$O) respectively. The dose selected was according to LD$_{50}$ of Na$_2$WO$_4$. 2H$_2$O as reported earlier in material safety data sheet (MSDS, 2006 b). Control rats (n = 6) were injected i.p. saline only. For urine collection, animals (n = 6 in each group) were randomly picked from all the four groups and kept in metabolic cages for three days for acclimatization. Urine samples from all the three tungsten treated groups were collected in ice-cooled tubes containing 1% sodium azide at 8, 16, 24, 72 and 120 h post dose (p.d.) whereas, for control (n = 6), urine samples were collected only once throughout the study. Supernatant was obtained by centrifugation, and then stored at -80°C for NMR spectroscopic analysis. Animals of low and high dose tungsten treated group and controls (n = 6 at each time) were sacrificed at 24, 72 and 120 h p.d. by cervical dislocation. Blood samples were collected from the orbital plexus in an eppendorf tube and allowed to clot for about 30 min. Serum samples were obtained for clinical biochemistry examinations using standard procedures. Both the kidneys and largest lobe of the liver was removed from each animal and weighed; duplicate samples from the largest lobe were excised, one whole kidney and one sample from the liver was fixed in 10% formalin for histopathological examination, the another kidney and liver was divided into 2 half, one half was used for antioxidant analysis and other half immediately snap-frozen in liquid nitrogen and stored at - 80 °C until NMR spectroscopic analysis.

6.2.2 Histopathology of liver and kidney
The histopathological procedure for liver is similar to that of kidney which has been described in Section 4.2.5 of Chapter 4.

6.2.3 Biochemical analysis of serum
The biochemical analysis has been described in Section 4.2.6 of Chapter 4.
6.2.4 Preparation of tissue homogenate
The preparation of liver tissue homogenate is similar to that of kidney which has been
described in Section 4.2.7 of Chapter 4. GSH, LPO, SOD and catalase assays carried
cut on tissue homogenates have been described in Section 4.2.7.1, 4.2.7.2,
4.2.7.3 of Chapter 4 and Section 5.2.4.1 of Chapter 5 respectively.

6.2.5 $^1\text{H}$ NMR spectroscopy of urine samples
The $^1\text{H}$ NMR spectroscopic measurement of urine has been described in Section
4.2.8 of Chapter 4.

6.2.6 $^1\text{H}$ NMR spectroscopy of serum
The $^1\text{H}$ NMR spectroscopic measurement of serum has been described in Section
4.2.9 of Chapter 4.

6.2.7 Extraction of polar metabolites in liver and kidney tissues
The extraction procedure of polar metabolites in liver is similar to that of kidney tissue
samples, which has been described in Section 4.2.10 of Chapter 4.

6.2.8 $^1\text{H}$ NMR spectroscopy of kidney and liver tissue extracts
The $^1\text{H}$ NMR spectroscopy of liver tissue extract is similar to that of kidney, which has
been described in Section 4.2.11 of Chapter 4.

6.2.9 NMR Data Processing and Multivariate Data Analysis
The NMR Data Processing and Multivariate Data Analysis have been described in
Section 5.2.9 of Chapter 5.
Additionally, to facilitate interpretation of the results, correlation coefficient of all
groups at each data point was obtained using metaboanalyst software. In this study, a
cutoff value of $r \geq 0.41$ ($r > 0.41$ and $r < -0.41$) was chosen for correlation coefficient
as significant based on the discrimination significance ($P<0.05$). Multivariate analysis
of NMR data was performed using a web-based Metaboanalyst program (Xia et al.,
2009).

6.2.10 Statistical analysis
The data obtained for concentration of serum and the antioxidant enzymes as well as
for the biochemical assays (LPO and GSH) were expressed as Mean ± SD. The
statistical significance of the inter group variation was measured by multivariate analysis of variance (MANOVA). Multiple comparisons using the Bonferroni Post Hoc test were performed to evaluate the discrimination significances between the control and the treatment group. Statistical significance was considered at $P < 0.05$.

6.3 Results

6.3.1 Histopathology and clinical biochemistry

The liver of control rats showed normal structure with centrilobular region. No sign of abnormality was observed (Fig. 6.1 a). In contrast, low and high dose treated groups showed degenerative changes in terms of slightly or distinctly blurred trabecular structure of the lobules, vacuolar degeneration, sinuses overfilled with blood. Changes observed were more prominent in high dose group treated animals and persisted till the end of the study. At 24 h the low dose tungsten treated group showed extensive degenerative changes in terms of fatty steatosis and sinuses overfilled with blood (Fig. 6.1 b), whereas the high dose group showed extensive trabecular blurring and sinuses overfilled with blood (Fig. 6.1 d). The damage to hepatic tissue was more prominent in high dose compared to low dose at 24 h. By the end of the study i.e. at 120 h, the hepatic tissue in low dose group showed signs of recovery (Fig. 6.1 c) whereas, the high dose treated group showed some degenerative changes and RBC in sinusoids (Fig. 6.1 e).

The kidney of control rats showed normal structure of cortex and medulla (Fig. 6.2 a). Moderate degenerative changes were observed in both the tungsten treated groups. Damage in terms of renal tubules hypertrophy i.e., tubular swelling (TS) and degeneration of epithelia of renal tubules (TD) was observed in both the dose groups at 24 h (Fig. 6.2 b and c). At the end of the study i.e., at 120 h p.d. low dose group showed signs of recovery (Fig. 6.2 d) but some changes still persisted in high dose group animals and (Fig. 6.2 e).

Clinical biochemistry results indicated that the rats treated with high dose of tungsten have higher level of serum ALT, AST at 24 h than controls, whereas the level of AST was found to be significantly lower in case of low and moderate
Fig. 6.1: Photomicrographs (200X) of liver sections with haematoxylin-eosin under light microscope (a) Control rats showing normal liver with centrilobular region. (b)(enlarged view at 400X) at 24 h Rats injected with low dose of Na₂WO₄Na·2H₂WO₂O₄ showing vacuolar degeneration.2H₂O showed extensive damage to, increased RBC in sinusoids liver tissue in terms of trabecular blurring and increased RBC in sinusoids (enlarged view at 400X) at 24 h. (c and e) Rats injected with low and high dose of Na₂WO₄.2H₂O showed increase in RBC in sinusoids and vacuolar degeneration only in high dose at 120 h respectively.
Fig. 6.2: Photomicrographs (200X) of kidney sections with hematoxylin-eosin under light microscope. (a) Control rats showing normal kidney with normal tubular brushthe tubules and glomerulus. (b and d) Rats injected with low dose of -borders (T) and intact glomeruli (G). No evidence of tubular inflNa$_2$WO$_4$.2H$_2$O showing tubular swelling (TS) and tubular ammation or congestion was found in degeneration (TD) at
24 h, which was recovered by 120 h (d). Photomicrograph (c) showed damage to kidney tissue in terms of tubular swelling and tubular degeneration, after administration of high dose of Na$_2$WO$_4$.2H$_2$O at 24 h and showed damage to continue to persist up to 120 h (e).
dose. At later time points i.e. at 72 and 120 h, AST was found to be significantly lower in all dose group, whereas ALT was lower only in case of high dose group at 120 h. Serum urea level was found to be significantly increased in both low and high dose group at 24 h, while at 72 h, increased level of urea was observed only in high dose group. Serum urea increased at moderate dose of tungsten at all time points. On the other side sporadic change was observed in creatinine level at 72 and 120 h in low and moderate dose respectively (Fig. 6.3).

6.3.2 Antioxidant status of renal and hepatic tissue from tungsten treated rats

To ascertain tungsten induced oxidative stress in renal and hepatic tissue, LPO and antioxidant enzymes such as SOD and catalase were measured. In addition, reduced GSH content was also measured. Tungsten induced oxidative stress was evident from significant increase in the level of LPO in liver at high dose upto 72 h as compared to controls. However, there was no significant change in renal LPO level after tungsten administration. Significant increase in GSH level was observed mainly in high dose group animals till 72 h in liver and till the end of the study i.e., by 120 h in kidney tissue. The more pronounced effect of oxidative stress induced by tungsten in liver has also been attributed from the significant increase in the level of SOD and catalase in both the dose group compared to controls. In case of renal tissue, not much change in the level of antioxidant enzyme was observed except for a significant increase in SOD level compared to control in low dose group at 24 h and significant increase in catalase level mainly in high dose group at 72 and 120 h p.d (Fig. 6.4 and 6.5).

6.3.3 Metabolite assignments with $^1$H NMR spectroscopy

A number of perturbations in endogenous metabolites were observed in the $^1$H NMR spectra of the urine and serum samples collected at various time points post dose (Table 6.1 and 6.2). Fig. 6.6 and 6.7 showed comparative $^1$H NMR spectra of urine and serum at 24 h as well as of urine at 120 h p.d (Fig 6.8) from control and different doses of Na$_2$WO$_4$. 2H$_2$O respectively. Spectral peaks of metabolites were assigned according to Lindon et al., (1999). In urine changes were observed in metabolites associated with energy metabolism (succinate, a-ketoglutarate (α-KG), citrate, acetate), gut flora metabolites [hippurate, tri-methylamine (TMA), dimethylamine (DMA)], osmolytes [trimethylamine oxide (TMAO), taurine],
Fig. 6: Doses of Na$_2$WO$_4$·2H$_2$O up to different time points of controls and * P< 0.05. Rats treated with low (40.8 mg), moderate (122.4 mg) and high dose (204 mg).
Fig. 6.4: Catalase, GSH, SOD and LPO level in liver tissue of rats treated with low and high dose of Na₂WO₄·2H₂O at different time points * P< 0.05.
Fig. 6: Different time points. 5: Catalase, GSH, SOD and LPO level in the kidney tissue of rats treated with low and high dose of Na\textsubscript{2}WO\textsubscript{4}·2H\textsubscript{2}O. Table 6.1: Significant changes in urinary metabolites of rats exposed to low, moderate and high dose of Na\textsubscript{2}WO\textsubscript{4}·2H\textsubscript{2}O compared to controls at different time points of treatment.
### Correlation coefficient, $R^2 > 0.41$, $P < 0.05$

**Table 6.2:** Significantly changed metabolites in the serum of rats exposed to low, moderate and high dose of Na$_2$WO$_4$.2H$_2$O compared to controls at different time points of treatment.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shift with multiplicity (Bracket)</th>
<th>8 h</th>
<th>16 h</th>
<th>24 h</th>
<th>72 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAA</td>
<td>0.9-1.05 (m)</td>
<td>-0.68</td>
<td>-0.90</td>
<td>-0.82</td>
<td>-0.80</td>
<td>-0.69</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.90 (s)</td>
<td>-0.74</td>
<td>-0.72</td>
<td>-0.77</td>
<td>-</td>
<td>-0.46</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>2.30 (s)</td>
<td>-0.65</td>
<td>-0.42471</td>
<td>-0.65</td>
<td>-</td>
<td>-0.64594</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>-</td>
<td>-0.80579</td>
<td>-0.75</td>
<td>-0.7148</td>
<td>-0.82034</td>
</tr>
<tr>
<td>N-acetylglutamate</td>
<td>1.89, 2.06 &amp; 2.1 (m)</td>
<td>-0.87</td>
<td>-0.86</td>
<td>-0.82</td>
<td>-0.75</td>
<td>-0.63</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.43(s)</td>
<td>-0.8</td>
<td>-0.71</td>
<td>-0.75</td>
<td>-0.82</td>
<td>-0.51</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>2.45(t) , 3.01(t)</td>
<td>-</td>
<td>-</td>
<td>0.90</td>
<td>0.58</td>
<td>0.43</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.55 &amp; 2.67 (AB)</td>
<td>-0.5</td>
<td>-</td>
<td>0.49</td>
<td>0.75</td>
<td>-</td>
</tr>
<tr>
<td>TMA</td>
<td>2.88 (s)</td>
<td>-0.65</td>
<td>-0.89</td>
<td>-0.66</td>
<td>-</td>
<td>-0.41</td>
</tr>
<tr>
<td>TMAO+Betaine</td>
<td>3.27 (s)</td>
<td>0.92</td>
<td>0.84</td>
<td>0.85</td>
<td>0.60</td>
<td>0.74</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.25 (t), 3.43 (t)</td>
<td>0.88</td>
<td>0.87</td>
<td>0.87</td>
<td>0.80</td>
<td>0.76</td>
</tr>
<tr>
<td>Hippurate</td>
<td>7.55 (t), 7.64 (t), 7.84 (d)</td>
<td>-0.47</td>
<td>-0.68</td>
<td>-0.62</td>
<td>-0.65</td>
<td>-0.45</td>
</tr>
<tr>
<td>Formate</td>
<td>8.46 (s)</td>
<td>-0.61</td>
<td>-0.83</td>
<td>-0.44</td>
<td>-</td>
<td>0.51</td>
</tr>
<tr>
<td>NMN</td>
<td>4.48 (s), 8.95, 8.97, 8.9 (d), 8.19 (t)</td>
<td>-</td>
<td>-0.87</td>
<td>-0.53</td>
<td>-0.60</td>
<td>-0.91</td>
</tr>
</tbody>
</table>
\[ R^2 = 0.71, \quad R^2 = 0.59, \quad R^2 = 0.88, \quad Q^2 = 0.40, \quad Q^2 = -0.14, \quad Q^2 = 0.61 \]

<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>Lipid (LDLs/VLDLs)</td>
<td>0.86, 1.30 (br)</td>
<td>-0.78636</td>
<td>-0.7389</td>
<td>-0.72378</td>
</tr>
<tr>
<td>BAA</td>
<td>0.94 (m)</td>
<td>0.92853</td>
<td>0.67778</td>
<td>0.53091</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>0.86118</td>
<td>0.64658</td>
<td>0.66993</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.03 (s)</td>
<td>0.86345</td>
<td>0.68572</td>
<td>0.81289</td>
</tr>
<tr>
<td>PEA</td>
<td>3.23 (t)</td>
<td>0.44324</td>
<td>0.68235</td>
<td>0.62591</td>
</tr>
<tr>
<td>TMAO+betaine</td>
<td>3.25 (s)</td>
<td>0.62911</td>
<td>0.39431</td>
<td>–</td>
</tr>
<tr>
<td>GPC</td>
<td>3.36 (s)</td>
<td>-0.86653</td>
<td>-0.84085</td>
<td>-0.88547</td>
</tr>
</tbody>
</table>

Correlation coefficient, \( r > 0.41, P < 0.05 \)
Fig. 6.6: Extended $^1$H NMR spectra of urine from control and rat exposed to low, moderate and high doses of Na$_2$WO$_4$.2H$_2$O at 24 h p.d. showing (a) aliphatic and (b) aromatic region.
Fig. 6.7: Extended $^1$H NMR spectra of serum from control and rat exposed to low, moderate and high doses of Na$_2$WO$_4$.2H$_2$O at 24 p.d.
Fig. 6.8: Extended $^1$H NMR spectra of urine from control and rat exposed to low, moderate and high doses of Na$_2$WO$_4$.2H$_2$O at 120 h p.d. showing (a) aliphatic and (b) aromatic region.
membrane metabolites (choline), amino acids (branched chain amino acids (BAA)), N-acetylglutamate (NAG), N-methylnicotinamide (NMN), creatinine and formate, whereas for serum changes were found mainly in the following metabolites viz lipids, BAA, alanine, creatine, Phosphorylethanolamine (PEA), TMAO and glycerophosphocholine (GPC). \(^1\)H NMR spectra of urine from Na\(_2\)WO\(_4\).2H\(_2\)O treated animals showed obvious alterations in the metabolite levels from 8 h time point onwards. Maximum changes were observed at 24 h time point mainly in high dose treated animals.

The \(^1\)H NMR spectroscopy of liver and kidney tissue extract was also performed in order to substantiate our understanding about multi-organ involvement. The \(^1\)H NMR spectroscopy of liver and kidney tissue extract of low and high were carried at different time points only in case of low and high dose of tungsten treated groups. Fig. 6.9 and 6.10 showed comparative \(^1\)H NMR spectra of liver and kidney tissue extract at 24 h p.d. from control and low and high doses of Na\(_2\)WO\(_4\). 2H\(_2\)O, respectively. Major endogenous metabolites for kidney and liver 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.\(^1\)H NMR spectroscopic analysis of liver and kidney homogenates revealed several metabolic changes at organ level after tungsten exposure in a dose dependent manner. To extract the detailed information about Na\(_2\)WO\(_4\).2H\(_2\)O induced metabolic alterations, multivariate data analysis of these NMR profiles was performed. Complete list of metabolites along with their chemical shift positions observed in different biological metrics are presented in Table (6.1, 6.2, 6.3 and 6.4)

6.3.4 Tungsten induced metabonomic changes

PCA analysis of urine, serum and tissue (liver and kidney) extracts was conducted to investigate the dose and time dose dependent effect of tungsten. The PCA score plot showed distinct clusters, which arose due to numerous differences in urinary, serum and tissue extract constituents between the control and tungsten treated groups. The PCA score plot of urine \(^1\)H NMR spectra showed clear separation of moderate and high dose groups, while the low dose group showed blended behaviour with controls at 8 and 16 h p.d (Fig. 6.11 a and 6.11 b). At 24 h p.d., 2D
Fig. 6.9: Extended $^1$H NMR spectra of liver tissue extract from control and rat exposed to low, moderate and high doses of Na$_2$WO$_4$.2H$_2$O at 24 p.d.
Fig. 6.10: Extended $^1$H NMR spectra of kidney tissue extract from control and rat exposed to low, moderate and high doses of Na$_2$WO$_4$.2H$_2$O at 24 p.d.
Table 6.3: Significantly changed metabolites in the liver tissue extract from rats exposed to low and high dose of Na$_2$WO$_4$.2H$_2$O compared to controls at different time points of treatment.

<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>Alanine</td>
<td>1.48(d)</td>
<td>−</td>
<td>0.54927</td>
<td>0.57671</td>
</tr>
<tr>
<td>Glu/Gln</td>
<td>2.15 (m)</td>
<td>−</td>
<td>0.61587</td>
<td>0.6434</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.40(s)</td>
<td>0.46701</td>
<td>−.59284</td>
<td>−</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.96 (m)</td>
<td>0.75493</td>
<td>0.82433</td>
<td>0.76094</td>
</tr>
<tr>
<td>Creatinine</td>
<td>3.04 (s)</td>
<td>0.74881</td>
<td>−</td>
<td>0.73576</td>
</tr>
<tr>
<td>Choline</td>
<td>3.20(s)</td>
<td>0.81279</td>
<td>0.94335</td>
<td>0.91826</td>
</tr>
<tr>
<td>PEA</td>
<td>3.23 (t)</td>
<td>0.84708</td>
<td>0.79563</td>
<td>0.87709</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.27(s)</td>
<td>−0.78539</td>
<td>−</td>
<td>−0.68694</td>
</tr>
<tr>
<td>Taurine</td>
<td>3.43 (t)</td>
<td>−0.78346</td>
<td>−0.60526</td>
<td>−0.64715</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.57 (s)</td>
<td>−</td>
<td>−0.81319</td>
<td>−0.80229</td>
</tr>
</tbody>
</table>

Correlation coefficient, ↑↑ 0.41, P < 0.05
Table 6.4: Na$_2$WO$_4$.2H$_2$O compared to controls at different time points of treatment. Ily changed metabolites in the kidney tissue extract from rats exposed to low and high dose of

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Chemical shifts</th>
<th>24 h</th>
<th>72 h</th>
<th>120 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>–</td>
<td>-0.60341</td>
<td>-0.67084</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.25 (s)</td>
<td>-0.48708</td>
<td>-0.54389</td>
<td>-0.61071</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.57 (s)</td>
<td>0.60671</td>
<td>0.68701</td>
<td>–</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>3.60 (s)</td>
<td>-0.66773</td>
<td>-0.90423</td>
<td>-0.7926</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>4.06 (t)</td>
<td>–</td>
<td>0.53514</td>
<td>0.62571</td>
</tr>
</tbody>
</table>

Correlation coefficient, $|r| > 0.41$, $P < 0.0$
score plot was not able to differentiate the treated groups but 3D score plot showed clear separation of high dose group from rest of the treated groups and controls (Fig. 6.11 c). At later time points i.e. at 72 and 120 h, all the three tungsten treated groups were still separated from control (Fig. 6.11 d and 6.11 e). On the other hand, the PCA score plot of serum $^1$H NMR spectra showed partial overlap of low dose group with that of control, while the moderate and high dose treated groups were clearly separated at all time points i.e. at 24, 72 and 120 h p.d (Fig. 6.12 a, b and c).

A total of 25 metabolites in urine and 12 metabolites in serum were identified based on loading scores of PCA. Relative integrals of identified metabolites were calculated and MANOVA showed significant difference in several metabolites. Based on $^1$H NMR spectra of serum and urine, it was observed that osmolytes, energy metabolites, gut flora metabolites, amino acids, membrane metabolites, formate, creatine, lipids and NMN were responsible for separation of tungsten treated groups from controls.

In addition to PCA, a supervised analysis PLS-DA based on these identified metabolites was performed for urine and serum to extract more useful information for segregation of treated groups from controls. As observed in PCA, PLS-DA also showed separation of treated groups from controls in urine sample (Fig. 6.13). However, at 24 h p.d, PLS-DA was better than PCA to resolve the separation of treated groups from controls in urine sample (Fig. 6.13 c). Likewise, PLS-DA score plot of serum showed classification of moderate and high dose tungsten treated group with partial overlap of low dose treated group with control at all time points (Fig. 6.14 a, b and c) as observed in PCA. To measure the robustness of the PLS-DA model, values for $R^2$ and $Q^2$ were used as indicators for the model fit in terms of goodness of fit and predictability of the model and the best separation among treated and controls groups was attained at 24 h p.d both in serum and urine based on PLSDA with $R^2$ and $Q^2$ values of 0.95 and 0.80 for urine and 0.71 and 0.40 for serum with 4th component as best classifier respectively (Table 6.1 and 6.2). Correlation analysis of different treated groups with controls identified 14 and 7 metabolites out of 25 and 12 metabolites used for PLS-DA analysis in urine and serum, respectively. The results of correlation analysis showed a significant decrease in the level of BAA, NAG, TMA, NMN, succinate, hippurate and formate, whereas the levels of α-KG, citrate, TMAO and taurine were found to be significantly higher in tungsten treated group compared to controls.
Fig. 6.11: The PCA score plot of full binned spectral region of urine (0.5-9.5 ppm) at (a) 8 h (b) 16 h (c) 24 h (d) 72 h and (e) 120 h p.d. from rats treated with Na₂WO₄.2H₂O at low, moderate and high dose and controls.
Fig. 6.12: The PCA score plot of full binned spectral regions of serum (0.5-4.5 ppm) at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low, moderate and high dose and controls.
Fig. 6.13: PLS-DA score plot based on $^1$H NMR spectra of urine sample at (a) 8 h (b) 16 h (c) 24 h (d) 72 h and (e) 120 h p.d from rats treated with Na$_2$WO$_4$.2H$_2$O at low, moderate and high dose and controls.
Fig. 6.14: PLS-DA score plot based on $^1$H NMR spectra of serum sample at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low, moderate and high dose and controls.
In case of serum samples a significantly decreased level of lipids and GPC with an increase in BAA, PEA, TMAO, alanine and creatine were found as compared to control group.

Besides biofluids, the effect of tungsten at organ level has also been determined using metabolic data analysis in low and high dose treated groups. The PCA plots of liver at 24 h (Fig. 6.15 a) showed clear separation of high dose treated group but low dose group was found to be partially overlapped with controls. However, at 72 h and 120 h both the treated were separated from control but treated group appeared to start approaching towards control by the end of the study (Fig. 6.15 b and c). The score plots of kidney also showed similar behaviour (Fig. 6.16). A total of 15 metabolites were identified in both liver and kidney based on loading scores of PCA. Relative integrals of identified metabolites were calculated and the concentration of each of the respective metabolites was than calculated and significant differences in several metabolites were identified using MANOVA. The result showed that the amino acids, membrane metabolites, osmolytes, succinate and creatinine were the main metabolites responsible for separation of treated group from that of the controls in both liver and kidney. However, serum biochemistry as well antioxidant parameters not showed much change in kidney tissue and reverted to normal by the end of the study whereas, $^1$H NMR spectroscopy depicted altered renal physiology. The score plots of PLS-DA of liver showed clear separation of high dose treated group, while control and low dose were partially overlapped at all time points (Fig. 6.17). The PLS-DA score plot of kidney showed blended behaviour of both the treated groups with that of controls at 24 h p.d (Fig. 6.18 a), whereas at 72 and 120 h only the high dose treated group was separated from the controls (Fig. 6.18 b and c). PLS-DA showed similar pattern of classification as observed in PCA in kidney. However, PLS-DA showed better separation of treated group from that of control in liver till the end of the study.

Correlation analysis of liver and kidney tissue extracts showed a significant decrease in alanine and TMAO compared to controls. Apart from this in liver the level of Glu/Gln, succinate, taurine were found to be significantly decreased, while creatinine, choline and PEA were found to be increased. In case of kidney glycine and myo-inositol were found to be decreased as compared to controls (Table 6.3 and 6.4).
Fig. 6.15: The PCA score plot of full binned spectral regions of liver (0.5-4.5 ppm) at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low and high dose and controls.
Fig. 6.16: The PCA score plot of full binned spectral regions of kidney (0.5-4.5 ppm) at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low and high dose and controls.
Fig. 6.17: PLS-DA score plot based on $^1$H NMR spectra of liver sample at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low and high dose and controls.
Fig. 6.18: PLS-DA score plot based on $^1$H NMR spectra of kidney sample at (a) 24 h (b) 72 h and (c) 120 h p.d. from rats treated with Na$_2$WO$_4$.2H$_2$O at low and high dose and controls.
6.4 Discussion

The aim of the present study is to investigate the metabolic consequences on exposure to tungsten using metabolomic approach. Though due to inert nature of tungsten, it is considered less toxic, some recent studies have shown some organ based toxicological effects on exposure to tungsten (Johnson et al., 2010). Therefore, the present study was planned to examine the acute toxic effect of different doses of tungsten at metabolic level in extensive biological matrices including serum, urine and tissue extracts (liver and kidney). The results reveal that exposure to tungsten lead to alteration in many of the metabolic pathways and tungsten exposure has potential influence on metabolic physiology of liver compared to kidney.

The underlying mechanism behind tungsten toxicity is the generation of reactive oxygen species (ROS) and affinity for thiol (-SH) group thus causing depletion of glutathione in various tissues (Tyrrell et al., 2013). Also tungsten in the form of phosphotungstate, disrupts the process of cellular phosphorylation and dephosphorylation (Johnson et al., 2010). Metals are known to alter the glycogen reserves and serum glucose levels by affecting the activities of liver enzymes that have role in the carbohydrate metabolism such as gluconeogenesis and glycolysis (Peiqiu et al., 2009). Hence, it is likely that tungsten also have an effect on carbohydrate metabolism in a similar way. Changes observed in the urinary metabolites associated with intermediary metabolism could be a result of tungsten induced toxic effect on liver metabolism. Changes in the level of Kreb cycle intermediates i.e., decreased succinate and increased citrate and α-KG content were observed in the present study. TCA cycle enzyme, aconitase is considered to be most sensitive enzyme for oxidative stress (Tretter and Adam-Vizi, 2000) and inhibition of aconitase has been suggested to be a sensitive marker of intracellular superoxide generation in mammalian cells (Gardner et al., 1995; Patel et al., 1996). This has been supported by significant increase in activity of SOD enzyme compared to control in liver tissue p.d. of tungsten treated animals. So there might be a possibility that inhibition of aconitase enzyme might have resulted in increased citrate level. In addition to aconitase, α – ketoglutarate dehydrogenase (KGDH) is also inhibited by high level of oxidative stress or ROS due to interaction with Fe-S clusters present at the catalytic site of the enzymes (Tretter and Adam-Vizi, 2000). The increase in the level of α-KG might be a result of accumulation of a KG due to inhibition of KGDH.

Further, it might have caused decrease in the level of succinate. Additionally, increased level of a KG and citrate in urine might be a result of altered renal tubular secretion and glomerular filtration. The decrease in the level of NMN in urine could be as a result of the antioxidant response triggered by tungsten induced oxidative stress. N-methylnicotinamide is a by-product,
formed during the conversion of Sadenosylmethionine (SAMe) to S-adenosyl homocysteine (SAH) and the Nmethyl nicotinamide so formed is then excreted out in urine. The SAH is further utilized in the transsulfuration pathway to regenerate GSH stores which are depleted in order to detoxify the reactive oxygen species (ROS) generated during tungsten exposure (Sun et al., 2008). Another consequence of oxidative stress induced by tungsten is the change in the cell integrity by the process of lipid peroxidation (LPO) (Yu, 1994; Miura et al., 1995). 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, have been evaluated as biomarkers of cytotoxicity (Hwang and Kim, 2007). The increase in the level of phosphorylethanolamine (a membrane lipid) along with decrease in the level of serum lipids and lipoprotein denoted the disruption of membrane fluidity caused by LPO. Similarly, oxidative stress induced changes have also been observed in liver in the form of increased level of phosphorylethanolamine and choline in $^1$H NMR spectra of liver tissue. It has further been supported by LPO and antioxidant enzyme assays as there was an increase in LPO in the liver specially in high dose treated group with concomitant increase in GSH as well the antioxidant enzyme i.e., SOD and catalase in order to counteract the toxic effect of tungsten. Changes in several amino acids in urine as well as in tissue extracts were also observed. The decrease in the level of urinary alanine along with its concomitant increase in serum suggests the effect of tungsten on the transamination reaction occurring in the liver. This has been further supported by decrease in the level of alanine in both renal and hepatic tissue extracts. Additionally, increased level of asparagin only in the liver tissue and altered serum biochemistry parameters supports the interference of tungsten with the transamination reaction occurring in the liver. The elevation of serum valine, leucine and isoleucine and reduced levels of these amino acids in urine further backs the enhanced catabolism involving BAA through transamination reaction leading to formation of ketone bodies. The decrease in the level of glutamine/glutamate in liver tissue extracts p.d. further support transamination reaction for the formation of αKG.

Besides TCA intermediates and amino acids, few other important metabolites are also present in urine spectra that reflect the influence of tungsten exposure on organ metabolism. Hippurate is another compound that is visible in urine spectra. Hippurate production is closely associated with microbiota associated metabolism. Decreased urinary levels of hippurate have been linked with dietary (Phipps et al., 1998) and intestinal microbiota changes (Gavaghan et al., 2001; Nicholls et al., 2003; Robosky et al., 2005). Production of hippurate from benzoate is a high energy consuming process, which
requires a good supply of ATP via oxidative phosphorylation. Reduced production of ATP due to the impaired TCA cycle might be responsible for the decreased level of hippurate in urine. Further, decreased hippurate levels in urine in the present study also indicate tungsten induced hepatotoxicity as liver plays an important role in the production of hippurate. The initial step of hippurate synthesis involves benzoate reaction with coenzyme A in hepatic cells. Tungsten toxicity induced oxidative stress might block the commencing step (due to high affinity to the -SH group), thereby resulting in decreased production of hippurate. In addition, the decrease in the urinary levels of hippurate might be correlated with poor intake or improper digestion of food. Another important metabolite that showed changes in liver and urine is taurine. The decreased taurine level in the hepatic tissue extract might be a consequence of enhanced membrane permeability due to tungsten led oxidative stress as a concomitant increase in taurine in urine has been observed post dose in treated group compared to controls. The elevated excretion of taurine has been suggested to occur via a number of mechanisms, and among these one of them is altered membrane permeability (Abebe and Mozaffari, 2011). Apart playing its role in maintaining the osmotic balance in various organs of the body, increased concentration of taurine in urine has been found to be related with hepatotoxic or non-hepatotoxic compounds induced disturbance in protein synthesis or sulphur amino acid metabolism compounds that may eventually lead to liver injury (Waterfield et al., 1993 a, Waterfield et al.,1993 b; Clayton et al., 2004). It is likely that tungsten might cause inhibition of protein synthesis or sulphur amino acid metabolism because of the high affinity between tungsten and thiolate (−SH), resulting in liver toxicity.

The present study revealed significant changes in renal tissue relatively in lesser number of metabolites compared to hepatic tissue. Most of the metabolites with significant changes in renal tissue were associated with maintenance of osmotic balance. A decrease in urinary level of TMA with concomitant increase in TMAO was observed till the end of the study. Further TMAO is produced from oxidation of TMA. It is one of the major medullary osmolytes required for maintaining renal osmolyte homeostasis (Garrod et al., 2001). The elevated level of urinary TMAO is a known marker of renal papillary lesion. These findings implied the tungsten induced renal papillary lesion, which was also confirmed by the alterations of biochemical parameters, the elevated serum urea and creatinine. Further the decrease in sarcosine level and increased myo-inositol in renal tissue suggests perturbation in ion regulation leading to tungsten induced osmotic stress. Weinberg et al., (1990) showed that during renal injury, amino acids acts as renal osmolytes against injury by maintaining cellular integrity. So it is speculated that change in the levels of osmolytes observed in renal tissue and
urine might be the body’s counter mechanism towards maintaining osmotic balance against tungsten exposure.

The results of both visual inspection of $^1$H NMR spectra of urine and serum as well the PCA and PLS-DA showed maximum alteration at 24 h p.d. especially in high dose treated group. Most of the changes in $^1$H NMR spectra of urine, serum and tissue extracts were found at 24 h, which might be due to the maximum toxic effect of tungsten till 24 h. Thereafter toxic effects of tungsten were found to be reduced, which might be due to the pharmacokinetics of tungsten having short retention time in the body with a half life of 24 h (Mcdonald et al., 2007).

6.5 Conclusion
The present study demonstrates the toxic effect of tungsten exposure in different biological matrices through metabolomic approach. Exposure to tungsten leads to perturbations in several metabolic pathways including lipid metabolism, perturbed TCA cycle, amino acid metabolism, gut flora metabolism and osmolytes. Tungsten exposure affected the hepatic physiology to a greater extent with maximum toxic effect at high dose. The results showed that an integrated NMR analysis of multiple biological matrices, such as urine, serum and tissue extracts samples, together with histological assessment, antioxidant parameters and clinical biochemical assays provided a comprehensive information on tungsten exposure mediated toxicity.