# NMR based metabolomic approach to study system responses of rat to tungsten exposure in multiple biological matrices

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**Target audience:** Researchers and students.

**Purpose:** Metals are well known environmental pollutants with hazardous effects on human health due to their wide usage in many industrial branches. They are present everywhere in the air, water and soils. Tungsten (W) is a complex metal that has numerous consumers, industrial and military application due to its tensile strength and high melting point. The toxicological effects of tungsten compared to other heavy metals have been poorly understood, but few recent incidents involving tungsten have raised concern for human and environmental safety. It has been suggested that oxidative stress is one of the main mechanisms involved in tungsten toxicity leading to the generation of reactive oxygen species (ROS), which in turn cause lipid peroxidation and protein oxidation in several tissues. Few research studies have reported the toxicity of tungsten at biochemical, enzymatic and genetic level, a comprehensive metabolome of tungsten toxicity remains to be elucidated. In recent years, NMR based metabolomics have been widely used for the assessment of toxic mechanisms, prediction of toxicity and identification of many organs based metabolite markers associated with heavy metal induced toxicity[1].

**Aim and Objective:** Identification of metabolic biomarker of acute toxicity induced by $Na_2W\ O_4.\ 2H_2O$ in multiple biological matrices (urine, liver and kidney) of rats using $^1$H NMR spectroscopy

**Material & Methods**: Male Sprague Dawley rats of 11 weeks of age (n = 5 in each group) and weighing 250-300g were injected with $Na_2W\ O_4.2H_2O$ [in 0.9% saline, at a dose of 40.8 (low), 122.4 (moderate) and 204 (high) $Na_2WO_4.2H_2O$ mg /kg body weight (b.w.)] intraperitoneally, controls were injected with 0.9% saline only. Urine samples were collected at 8, 16, 24, 72, and 120 h post dose (p.d.) and placed at -80$^0$C till NMR Spectroscopy was carried out. Another set of animals were sacrificed at 24, 72 and 120 h p.d. and blood and tissue (liver and kidney) samples were collected. Kidney and liver were excised, washed with phosphate saline buffer and immediately snap frozen in liquid nitrogen. Polar metabolites from kidney and liver tissues were extracted using perchloric acid and the supernatant containing metabolites was lyophilized for 8-10 hours. For urine samples 400µl of centrifuged urine sample was added to 200 µl of deuterated phosphate buffer (pH= 7.4) containing 1mM TSP whereas the lyophilized tissues were reconstituted in 600µl of $D_2O$ containing 1mM TSP and all the samples were transferred to 5mm NMR tubes. $^1$H NMR spectra were acquired at 400.13 MHz, Bruker-AVANCE 400 spectrometer at 298K. 1D NOESYPR and 1D ZGPR pulse sequence was used to achieve satisfactory water suppression for urine samples and aqueous extracts respectively. Typically 64 scans were acquired with a relaxation delay of 2 s, flip angle of 90° and spectral width 10 ppm. All data sets were zero-filled to 32K data points and FID was weighted by an exponential function with a 0.3Hz line broadening prior to Fourier transformation. Peak assignment was determined according to previously reported literature[2]. NMR spectra were segmented into region of 0.04 ppm width. The area for each segmented region was calculated and normalized to the total spectral area of each $^1$H NMR spectrum. In order to discern the presence of inherent similarities of spectral profiles and to order recognize the spectral pattern of endogenous metabolites related to $Na_2W\ O_4.2H_2O$ multivariate analysis using metaboanalyst (http://www.metaboanalyst.ca/Metaboanalyst/faces/Home.jsp) was carried out. The blood samples were allowed to clot and serum was obtained by centrifugation at 2655 g for 10 minutes. Biochemical assays such as SGOT (serum glutamate oxaloacetate transaminase), SGPT (serum glutamate pyruvate transaminase), urea and creatinine were also carried out in serum samples.

**Results:-** A number of alterations in endogenous metabolites were observed in $^1$H NMR spectra of urine and tissue extract samples collected at different time points. Figure 1 and 2 shows representative NMR urine spectra and PCA plots at 24 h p.d. repesctively. PCA score plots of urine, liver and kidney tissue samples showed a clear separation between controls and treated groups. The urine spectra were dominated by a number of metabolites associated with energy metabolism (succinate, citrate, α – Ketoglutarate (α – K.G), trans-aconitate, lactate), gut flora metabolites (hippurate), osmolytes (taurine, TMAO), amino acids [Branched chain amino acids (BAA), phenylalanine], N-acetylglutamate (NAG), N-methyl nicotinamide, creatinine, Glycine, sarcosine, formate and Dimethylamine (DMA). For liver and kidney tissue samples most of the changes were observed in creatinine, choline, Phosphorylethanolamine (PEA), TMAO, taurine, glu/gln, sarcosine and myo-inositol. In urine samples most of the metabolites reverted to normal level, except for the metabolites which have shown changes throughout the study viz. energy metabolites, hippurate, TMAO, taurine and N-methyl nicotinamide. In case of kidney tissue most of the changes came back to normal in all the dose groups by 120 h p.d. whereas for liver tissue changes still persisted till 120 h only in case of high dose group treated animals. Maximum toxic effects of tungsten were observed at 24 h p.d mainly in high dose treated groups in both urine and tissue extracts

**Discussion:** - Tungsten is known to cause oxidative stress and in the form of tungstate, it polymerizes with phosphate which might cause disruption of cellular phosphorylation and dephosphorylation process[3].The increase of TCA intermediates in urine after tungsten exposure might be due to altered renal tubular physiology. Tungsten exposure showed prominent changes related to hepatotoxicity in urine spectral profiling as taurine, a potent marker of hepatotoxicity showed a time and dose dependent change. This has been very well supported by the alteration in the biochemical parameters i.e. SGOT and SGPT in serum. Also the decrease in the urinary hippurate level present only in the case of the high dose group might be related to tungsten induced liver toxicity as liver plays an important role in the production of hippurate. Reduced production of ATP due to impaired TCA cycle might also be responsible for decreased levels of hypocrite and it requires a large supply of ATP. The increase in the level of urinary choline might be due to disturbance of cellular integrity as a result of oxidative stress induced by tungsten. Choline is formed from lecithin via phosphocholine by enzyme choline oxidase. Tungsten might cause inhibition of the enzyme choline oxidase resulting in increased excretion of choline. The decrease in the level of N-methyl nicotinamide mainly in moderate and high dose group might suggest glutathione based detoxification reaction occurring in the body for removing free radicals generated during oxidative stress caused by tungsten. The elevated urinary TMAO level in a dose dependent manner is a known marker of renal papillary lesions[4]. This has also been supported by elevated serum clinical level of urea and creatinine. The damage to both liver and kidney was also seen as altered level of various metabolites in the extracts of liver and kidney along with changes in the urinary metabolic profile. The changes were more prominent in liver till the end of the study, which might be due to a maximum accumulation of tungsten in the liver because of the high concentration of molybdenum-dependent enzymes (e.g., xanthine oxidase [XO]), which tungstate competes for the binding site, rendering the enzymes inactive[3]. The outcome of the study is that tungsten affects both renal as well as hepatic physiology and also many other metabolic pathways and the perturbations can be seen as early as 8 h p.d.

**Conclusion:** NMR-based metabolomics has been proved to be an efficient and valuable technique for the metabolic marker identification of acute toxicity of metals. The $^1$H NMR based metabolomics approach can provide a systematic and holistic view of the biochemical effect of tungsten toxicity.

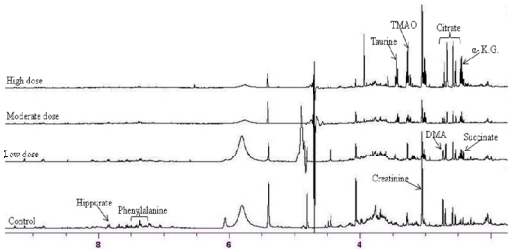


Fig.1 Representative $^1$H NMR urine spectra from control and animals injected with low, moderate and high doses of $Na_2WO_4.2H_2O$ at 24 h

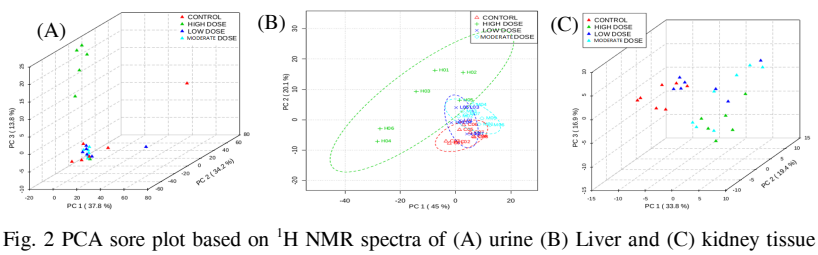


Fig. 2 PCA sore plot based on $^1$H NMR spectra of (A) urine (B) Liver and (C) kidney tissue samples at 24 h from controls and animals injected with low, moderate and high doses of $Na_2WO_4.2H_2O$.

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