Austin Journal of Analytical and Pharmaceutical Chemistry

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Austin Publishing Group

Research Article

Evaluating Metabolic Responses in Mice to Nanosized Titanium Dioxide Particles Using Gas Chromatography-Mass Spectrometry Based Metabolomics

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Received: April 20, 2017; **Accepted:** May 18, 2017; **Published:** May 25, 2017

Abstract

Titanium dioxide nanoparticle (TiO_2 NP) is one of the most commonly used engineered nanoparticles. It has attracted lot of interest to analytical toxicologists in recent past due to its toxicity on human health and environment. The present study was aimed to explore the GC-MS based metabolomics as a tool to investigate the toxicity of TiO_2 NP in comparison to TiO_2 BP with doses of 300, 600, 1200 mg/kg respectively in both gender(s) of Swiss Albino mice for 7 and 14 days. Serum biochemistry and histopathology parameters were performed. Chemometric analysis by supervised PLS-DA was performed to identify the discrimination/classification between exposed and non-exposed samples due to metabolic perturbations. The morphological, biochemical, haematological and metabolomic analysis revealed that, TiO_2 NP has induced toxicological effects to both female and male mice. The results showed that metabolomics along with biochemical analysis can be employed as a comprehensive tool to identify the toxicity of NPs in the model organisms at molecular level.

Keywords: Toxicity; Nanoparticles; Gas chromatography-Mass Spectrometry; Metabolomics

Abbreviations

GC-MS: Gas Chromatography-Mass Spectrometry; NP: Nano Particles; BP: Bulk Particles; TiO_2 NP: Titanium Dioxide Nano Particles; TiO_2 BP: Titanium Dioxide Bulk Particles; PLS-DA: Partial Least Square Discriminant Analysis; NMR: Nuclear Magnetic Resonance; AMDIS: Automated Mass Spectral Deconvolution and Identification System; NIST: National Institute of Standards and Technology; MS: Mass Spectrometry

Introduction

Titanium dioxide nanoparticles (TiO_2 NPs), one of the most widely engineered nanoparticles has many industrial applications in the areas of cosmetics, drug delivery, pigment in paints, ceramics and pharmaceuticals [1,2]. Globally, TiO_2 NPs are abundantly produced and widely used because of their smaller size and larger surface area with high stability and anticorrosion properties. Nowadays, a large number of nanoparticles are entering into our environment due to their usage due to advances in nanotechnology, thus, causing concern as they have potential impacts on human and environmental health [3]. The conventional toxicological experiments has revealed that, TiO_2 NPs can produce free radicals with strong oxidizing ability which thus induce oxidative stress and finally resulted in apoptotic cell death, fibrosis, DNA damage and pulmonary inflammation [4-6].

In recent years, metabolomics has been shown as a valuable tool to identify and quantify the global changes in small molecular weight metabolites (amino acids, organic acids, sugar, fatty acids etc.,) of an intra-cellular system to therapeutic intervention or toxicant and diseases [7]. Thus, metabolomics is considered as a potential tool in functional genomics, disease diagnosis, toxicology and pharmacology research [8,9]. This approach has been successfully used in toxicological sciences to understand the mechanism of action and to identify the biochemical responses to toxicant exposure.

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are considered to be two complementary analytical platforms to study the metabolic responses in any organism [10,11]. The separation efficiency and identification potential of mass spectrometry based metabolomics have improved by coupling it with separation techniques like gas and liquid chromatography [12-15]. The gas chromatography-mass spectrometry (GC-MS) based metabolomics has various advantages over other hyphenated analytical techniques which include, high chromatographic resolution, analyte-specific detection and quantification as well as capability to identify unknowns made it a suitable tool for metabolomics in the fields of toxicity and biomarker discovery. Therefore, the study has been carried out to evaluate the molecular events following oral dose of nanosized and bulk sized TiO_2 particles in Swiss Albino mice at three different doses 300, 600 and 1200 mg/kg body weight for 7 and 14 days by using GC-MS combined with pattern recognition approaches. Serum biochemistry and haematology tests were also performed.

Citation: Singh AK, Ratnasekhar Ch, Chaudhari BP, Singh D, Chattopadhyay BD, Mudiam MKR. Evaluating Metabolic Responses in Mice to Nanosized Titanium Dioxide Particles Using Gas Chromatography-Mass Spectrometry Based Metabolomics. Austin J Anal Pharm Chem. 2017; 4(2): 1085.

Materials and Methods

Chemicals and reagents

All chemicals used were analytical grade. Methoxyamine hydrochloride, N-methyl-N-trimethylsilyl trifluoracetamide (MSTFA) and all standards were procured from Sigma- Aldrich (St. Lous, MO, USA). Methanol was obtained from Sigma Aldrich (St. Lous, MO, USA). The ultra-pure water was prepared by RiOsTM water purification system (Millipore, Billerica, MA, USA). IMECO ULTRA SONICS (Bombay, India) was used as sonicator. Heto GD-2 maxi dry plus (Germany) was used as lyophilizer.

Particle characterization

A stock solution of 1mg/ml of TiO_2 NP in Milli-Q water was prepared and subjected to 15 min ultrasonic vibration (Sonics & Material Inc.) for dispersion. Furthermore, the surface morphology of the TiO_2 NP was confirmed by using a scanning electron microscope (SEM with EDAX – Apollo XL, FEI, Eindhoven, Netherlands).

Animal selection

The present study was carried out on male and female Swiss Albino mice weighing 25-30gm. The animals were housed in polypropylene cages with stainless steel grids under optimal conditions (humidity 50 ±10%), temperature 22 ±3^{0}C and light intensity 12-h light/dark cycle). Animals were provided with fed water and standard pellet diet *ad libitum*. The study protocol was approved by institutional ethics committee at CSIR-IITR.

Experiment design

Adult female and male mice were divided into 13 groups ((six groups for 7 days, six groups for 14 days and one control groups). Each group consists of 5 male and 5 female animals. These groups were given 300, 600, and 1200 mg/kg TiO_2 NP and TiO_2 BP administered by a single oral gavage according to the Organization for Economic Co-operation and Development, 420 (OECD, 1992) to Swiss Albino female and male mice for 7 and 14 days. Body weight and clinical signs of toxicity were recorded throughout the period of experiment [16,17].

Sign of toxicity and mortality

Signs of toxicity such as diarrhea and body weight loss were observed once daily throughout the experiment. After 7 and 14 days of dosing mice were sacrificed and blood was collected in 10% ethylene diamine tetra acetic acid (EDTA) anticoagulant containing tubes for hematology analysis and non-oxalate tubes for the separation of serum for metabolomic and biochemistry analysis.

Clinical biochemistry

The clinical biochemistry parameter of serum samples were carried out with fully automated biochemical analyzer (Clinical chemistry analyzer Randox-daytona UK) using standard kits. The following parameters were tested: glucose (GLU), creatinine (CREA), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), triglycerides (TG), alkaline phosphate (ALP), uric acid (URCA) and cholesterol (CHOL).

Hematological parameters

Blood collected in 10% EDTA was analyzed for white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), neutrophils (NEUT) lymphocyte (LYM), monocyte (MONO), eosinophils (EO) and basophils (BASO) through automated cell counter Hematology analyzer (Sysmex XT-2000iV Analyzer-IDEX America) using standard kits.

Metabolite extraction and derivatization

Extraction of metabolites from serum and derivatization was implemented in a similar approach as previously described with little modifications [18]. Serum metabolites were extracted by adding 800µl of MeOH (80%, *v/v*) for deproteinization. After vortexing, samples were centrifuged at 10,000rpm for 15min. Then the extraction procedure with 80% MeOH was repeated thrice and then all supernatants were pooled. The pooled extract was freeze dried using Scanvac system (Labogene, Denmark). To the resultant residue, an amount of 40µl of methoxyamine hydrochloride was added and mixed vigorously for 1min and then incubated at 65°C for 30min. To this, 90µl of N-methyl- N-(trimethylsilyl)-trifluoroacetamide was added and incubated at 65°C for 1hr under agitation using a thermo mixer (BR BIOCHEM Life sciences, India). Samples were made up to the volume of 400µl using hexane for further analysis using GC-MS.

GC-MS instrumentation and data acquisition

The GC-MS analysis was performed for metabolomics as previously described in studies [19]. Metabolite profiling was performed on Trace GC ultra (Thermo Scientific, FL, USA) coupled to TSQ Quantum XLS mass spectrometer (Thermo Scientific, FL, USA). TG-5MS fused silica capillary column (30m x 250µm i.d; Thermo Scientific), chemically bonded with 5% phenyl 95% methyl polysiloxane cross linked stationary phase (0.25µm film thickness) was utilized to separate the peaks. GC injector was used in split less mode. The injector temperature was set at 260^{0}C. Helium, the carrier gas, was maintained at a constant flow rate of 1.1ml/min during the analysis. The column temperature was initially kept at 65^{0}C for 2min, then ramped to 230^{0}C at a rate of 6^{0}C/min and then finally increased to 290^{0}C at a rate of 10^{0}C/min, where it was held for 20min. The interface temperature and ion source were set at 290^{0}C and 220^{0}C respectively. Electron impact ionization (EI^+) mode was used for mass detection with electron energy of 70eV. Mass spectra were acquired with a scan range of m/z 45-800. Solvent delay was set at 7min. The sample volume of 1µl was injected in GC-MS for analysis [19].

Data pre-processing

Serum used for metabolomic analysis as a single batch in random order. AMDIS software (version 2.0) was used to identify the metabolites in serum. The mass spectra of all the detected compounds were compared with spectra in NIST library (version 2.0) or standards for confirmation. All GC-MS raw data files were exported into Net CDF format using XCalibur software (Thermo Fisher Scientific, FL, USA).

Deconvolution of the Net CDF format files was performed using the XCMS software [20]. The data was arranged on a three dimensional matrix consisting of arbitrary peak index (RT-m/z pair), sample names and peak area. The total area normalization was performed in order to reduce the systematic biases within the experiment. Normalized data was used for multivariate analysis to remove the offsets and adjust the importance of high and low

Table 1: Morbidity and mortality in mice after orally administered TiO_2 NPs.

Dose (mg/kg)	Body weight (g)			Total deaths	Signs of toxicity
	0 days	7 days	14 days		
Female					
Control (n=5)	25.3±0.8	27.3±0.8	31.8±0.8	0	Nil
300 (n=5)	26.2±0.7	28.3±0.7	32.2±0.7	0	Nil
600 (n=5)	25.4±0.6	27.5±0.6	33.5±0.8	0	Nil
1200 (n=5)	27.6±0.7	28.9±0.7	26.2±0.9	0	Diarrhea, weight loss
Male					
Control (n=5)	25.8±0.8	26.9±0.6	32.8±0.7	0	Nil
300 (n=5)	25.7±0.9	26.7±0.7	33.2±0.9	0	Nil
600 (n=5)	26.6±0.7	27.4±0.3	33.5±0.6	0	Nil
1200 (n=5)	26.8±0.5	28.6±0.6	25.2±0.5	0	Diarrhea, weight loss

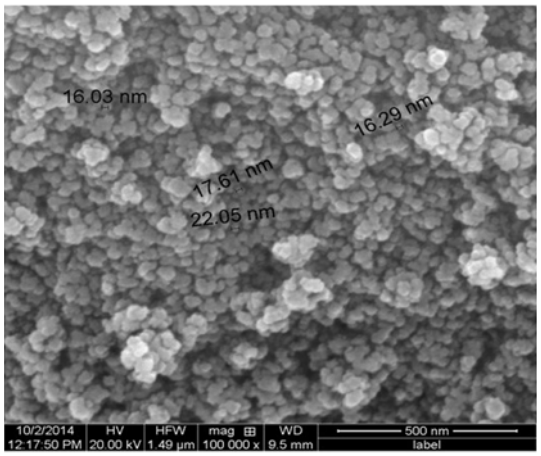

Figure 1: SEM image of TiO_2 NP (< 25 nm).

abundance metabolites to an equal level.

Multivariate pattern recognition analysis

Metabo Analyst tool was used for data processing and statistical analysis [21]. To identify the differential metabolites that account for the separation between groups, supervised PLS-DA was applied. PLS-DA model was validated using the leave one out cross validation method [22]. Quality of model is assessed on R^2 and Q^2 scores [23]. Further, model validation was performed by 500 times permutation tests [24]. Metabolites with variable importance in projection (VIP) values of greater than 1 were taken as potential marker metabolites.

Results and Discussion

In-life parameters

Oral administration of TiO_2NP at 300 and 600 mg/kg did not produce any signs of toxicity and mortality during 7 and 14 days exposure in both male and female mice. However, there was significant reduction in body weight of animals at high dose (1200mg/kg) at 14 days along with significant toxicity symptoms (Table 1).

Characterization of TiO_2 NPs

Characterization of NPs is one of the critical factors responsible for nanoparticles property and their mechanism of cellular interaction. We also measure the average size of NPs by scanning electron microscope (SEM). It was ranged from 16.03 to 22.05 nm (Figure 1).

Animal observation and body weight

Adverse signs, symptoms and mortality were not observed after 7 and 14 days of oral treatment with 300, 600 and 1200 mg/kg BW of nanosized and bulk sized TiO_2 particles in mice. However, mice treated with high dose (1200mg/kg) of TiO_2 NPs showed some irritation, dullness and the significant reduction in feed intake, body weight gain.

Clinical biochemistry

The results of serum biochemical parameters of female and male mice after orally administered TiO_2 NP for 14 days were shown in Table 2. Serum biochemistry parameters such as Glucose, AST, ALT, triglycerides and alkaline phosphate were significantly changed in both female and male mice exposed to TiO_2 NP at high concentration (1200mg/kg) dose as compared to controls for 14 days. No changes were observed in biochemistry parameters after exposure to

Table 2: Biochemical data of mice orally administered TiO_2NP for 14 days.

	TiO_2 NP(mg/kg)						
Parameters	Control	300	600	1200	300	600	1200
		Female			Male		
GLU (mg/dL)	120.47±7.51	125.00±2.65	127.67±6.12	127.67±5.78	129.67±4.48	115.33±2.02	105.33±1.85*
CREA (mg/dL)	0.37±0.06	0.40±0.06	0.39±0.10	0.38±0.03	0.43±0.06	0.48±0.15	0.37±0.03
AST (µ/L)	176.00±23.26	180.00±7.94	198.33±9.21	210.00±17.50	251.33±12.57	277.67±20.51	298.67±9.83*
ALT (µ/L)	177.00±4.93	182.33±16.22	178.00±3.21	164.10±0.95	175.67±4.41	190.33±3.67	219.33±4.70*
TP (g/dL)	6.37±0.45	4.27±0.09	4.53±0.18	5.03±0.22	4.50±0.35	5.20±0.04	5.37±13.81
TG (mg/dL)	50.67±2.60	56.67±7.54	54.00±6.51	57.00±2.52	53.63±0.35	51.33±3.38	44.33±5.69*
ALP (µ/L)	373.67±38.20	345.67±6.74	354.67±12.33	354.67±0.33	382.00±10.26	409.67±46.96	416.33±16.49*
URCA (mg/dL)	1.37±0.15	0.57±0.18	0.50±0.15	0.83±0.28	1.43±0.18	14.33±1.20	5.77±4.11
CHOL (mg/dL)	60.67±6.23	61.67±0.33	58.67±5.36	55.33±1.76	61.00±7.81	58.67±3.38	40.00±1.00
GLU (mg/dL)	120.47±7.51	125.00±2.65	127.67±6.12	127.67±5.78	129.67±4.48	115.33±2.02	105.33±1.85*

*Significant at the level of $p < 0.05$.

Table 3: Hematology data of mice orally administered TiO_2 NP for 14 days.

	TiO_2 NP (mg/kg)						
Parameters	Control	300	600	1200	300	600	1200
		Female			Male		
WBC (10^3/µL)	14.07±4.81	17.04±0.76	16.08±0.86	15.02±0.91	16.36±0.75	19.99±3.71	26.91±2.86
RBC (10^6/µL)	5.93±0.45	6.19±0.31	5.17±0.45	5.67±0.47	5.64±0.52	6.15±0.69	6.46±0.64
Hb (g/dl)	11.93±0.55	10.80±1.51	11.37±2.17	13.33±0.88	10.13±0.95	13.20±0.80	10.83±2.00
HCT (%)	37.97±1.55	37.90±1.42	32.70±3.08	39.60±1.80	31.67±3.01	33.23±1.52	29.20±5.90*
MCV (fl)	64.50±4.22	62.07±2.99	67.47±4.21	81.50±6.21	62.93±0.44	63.00±5.03	57.50±1.12
NEUT (%)	9.97±1.93	10.00±2.98	13.97±3.25	14.87±4.79	15.20±2.55	14.67±2.35	24.43±1.63*
EO (%)	0.90±0.15	1.17±1.82	1.23±052	1.97±0.32	1.87±0.91	1.90±2.41	0.77±0.12
BASO (%)	0.83±0.55	0.67±0.33	1.00±0.31	0.80±0.40	0.63±0.15	0.30±0.10	1.03±0.60*
LYM (%)	84.03±3.68	64.77±1.34	79.03±3.81	68.77±8.10	81.50±4.25	61.73±12.09	108.00±10.75*
MONO (%)	4.27±1.30	5.07±0.75	5.13±0.29	4.07±0.39	3.80±0.76	3.40±0.64	4.23±1.57

*Significant at the level of $p < 0.05$.

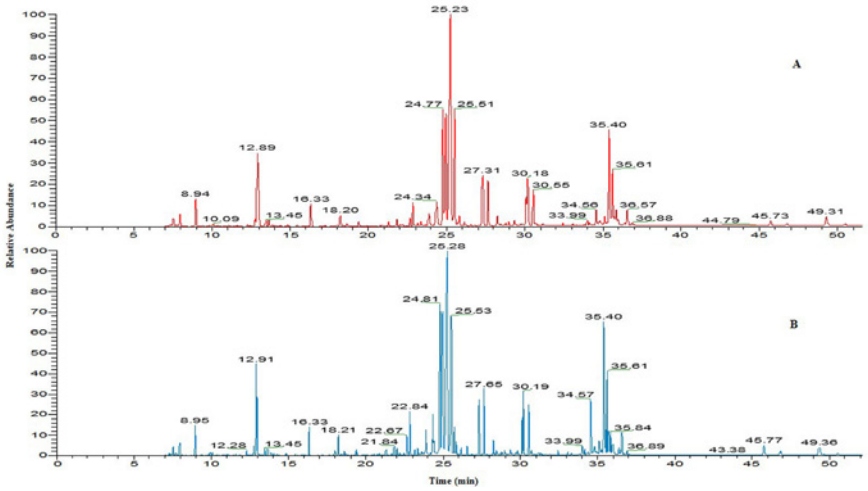

Figure 2: The total ion chromatogram of serum samples of (A) control; (B) 1200 mg/kg TiO_2 NP exposed mice.

TiO_2NP for 7 days. In the study, we found increased levels of serum enzymes AST, ALT and alkaline phosphatase in treated mice at high concentration (1200mg/kg) of TiO_2 NP at 14 days. It is considered to be potential biomarker of liver damage. Therefore, our data suggest that liver damage might have occurred in mice due to exposure of TiO_2 NPs.

Haematological parameters

There were no significant changes in haematological parameters in both gender orally administered to TiO_2 NP and TiO_2 BP (300,600,1200 mg/kg) for 7 days. However, a significant changes ($p<0.05$) were noted in WBC, HCT, NEUT and BASO in animals after exposed to TiO_2 NP (1200mg/kg) as compared to control for 14 days (Table 3). WBC, HCT, NEUT and BASO significant increased in TiO_2 NP treated mice at high dose (1200mg/kg) for 14 days, suggesting that nanosized TiO_2 particles may induce inflammation and it may also generate oxidative stress via activation of oxidative stress responsive transcription factors [25]. Therefore increased level of some haematology parameters like WBC resulting from administration of TiO_2 NPs may induce oxidative stress. Earlier studies also reported that TiO_2 NP can produce free radicals which can exert a strong oxidizing ability and produce oxidative stress in rodents [26-29].

Metabolic responses of mice after exposure to TiO_2 NP

All mice were survived in the experimental conditions and no mortality was found in treated group of mice after exposure for a period of 7 and 14 days. The results of metabolomic studies were carried out at three different doses 300, 600, 1200 mg/kg BW of TiO_2 NP respectively. Figure 2 shows that a representative total ion chromatogram of serum of control and exposed mice at high concentration (1200mg/kg) of TiO_2 NP.

Multivariate analysis was performed to reduce the data to low dimensional space, where discrimination of metabolic profiles between sample classes can be modelled. Supervised PLS-DA was performed to find a small number of linear combinations of the original variables, which was predictive for the class membership and that, described most of the variability of the metabolic profiles of control and exposed samples. PLS-DA loadings plots were used to identify the metabolites that were responsible for the observed

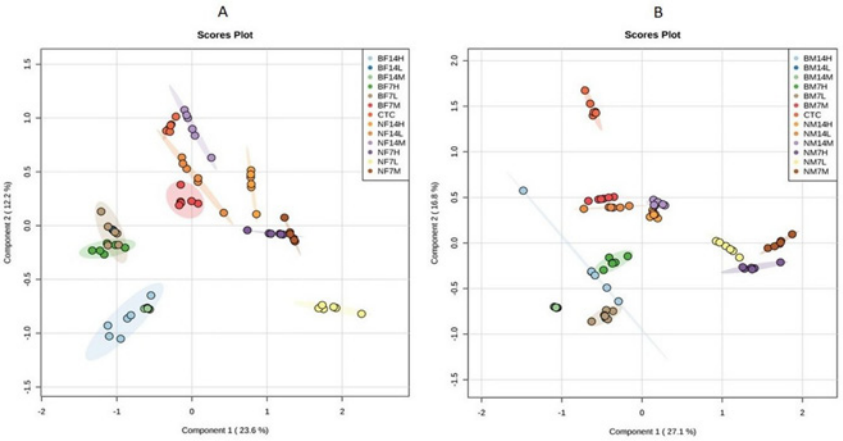

Figure 3: Partial least square discriminate analysis (PLS-DA) scores plot of first component Vs second component for (A) female mice; (B) male mice extracts showing the separation of control group, from TiO_2NP and TiO_2 BP exposed mice for 7 and 14 days at concentration of 300 mg/kg (L; Low dose), 600 mg/kg (M; Medium dose), 1200 mg/kg (H; High dose). BF14; Bulk particle in female mice for 14 days, BF7; Bulk particle in female for 7 days, CTC; control, BM14; Bulk particle in male mice for 14 days, BM7; Bulk particle in male mice for 7 days, NF14; Nanoparticle in female mice for 14 days, NF7; Nanoparticle in female mice for 7 days, NM14; Nanoparticle in male mice for 14 days, NF7; Nanoparticle in male mice for 7 days.

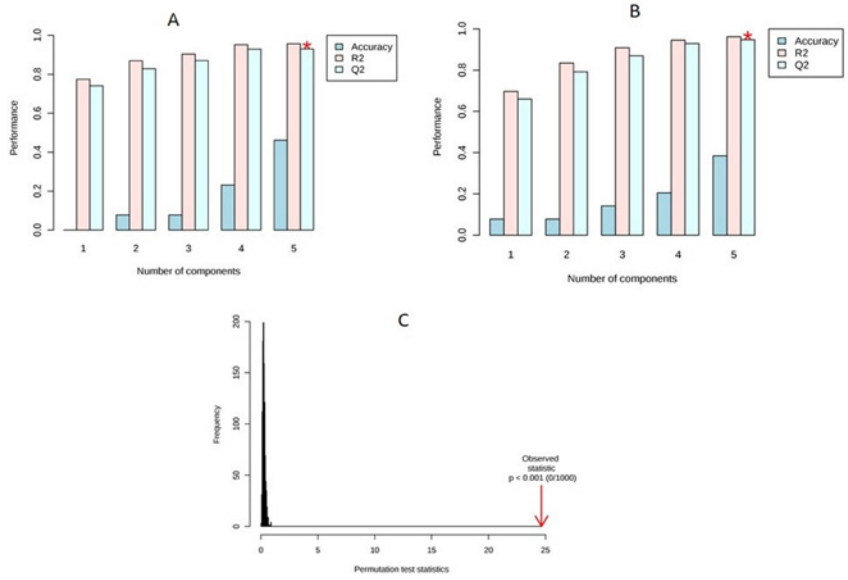

Figure 4: (A) Cross validation of female mice; (B) Cross validation of male mice; (C) Permutation analysis of PLS-DA models derived fromTiO_2 exposed and controls of female and male mice.

separation between scores of the control and treated mice. The PLS-DA results were displayed as scores plots indicating scatter of samples, which indicate similar metabolomic compositions when clustered together and compositionally different metabolomic compositions when dispersed. In female mice, the supervised PLS-DA model obtained from GC-MS analysis of the samples revealed that the general structure of the complete data set, in which two components cumulatively accounted for 35.8% of the total variance with Component 1 (C1) explained 23.6% and Component 2 (C2) explained 12.2% (Figure 3A).

In male mice, two components cumulatively accounted for 43.9% of the total variance with C1 explained 27.1% and C2 explained 16.8% of the total variance respectively (Figure 3B). In both male and female mice, clear clusters were identified in PLS-DA scores plot. PLS-DA scores plot showed that, there was significant separation ($p<0.05$) among control and TiO_2 NP and TiO_2 BP treated group of mice along first component. A clear, linear dose trend with separation becoming more obvious the greater the dose (Figure 3). According to obtained results from PLS-DA, it is clearly suggested that changes of metabolic responses in treated mice were concentration dependent.

Visual examination of PLS-DA score plots is not a reliable method for determining predictive power. Therefore internal cross validation was performed to find out the predictive accuracy and fit of the polynomial model. The cumulative values of PLS-DA model with R^2X_{cum} = 0.349, R^2Y_{cum} = 0.431, Q^2X_{cum} = 0.297, Q^2Y_{cum} = 0.352 shows good fit of the model in female mice (Figure 4A). The cumulative

Table 4: Differential metabolites in female mice between TiO_2 NP exposed and control groups.

S.No	CheBI ID	Name of metabolite	RT (min)	*m/z*	VIP score	*p*- value	Content variance
1	CID:22600307	Silanamine	15.16	188	1.0218	5.10E-30	Decreased
2	CHEBI:6650	Malic acid	17.92	245	1.3999	8.73E-34	Increased
3	CHEBI: 18183	Pyroglutamic acid	18.47	156	1.5802	9.20E-30	Decreased
4	CHEBI:30915	Ketoglutaric acid	19.62	288	1.0597	1.03E-17	Decreased
5	CHEBI:18257	Ornithine	22.81	174	1.6673	8.25E-35	Increased
6	CHEBI:30887	Isocitric acid	24.21	465	1.7452	1.27E-27	Increased
7	CHEBI:28875	Tetradecanoic acid	24.30	285	1.7662	4.85E-26	Increased
8	CHEBI: 18186	Tyrosine	25.05	179	1.554	6.85E-24	Increased
9	CHEBI:28757	Glucose	25.36	217	1.054	2.01E-14	Decreased
10	CHEBI:59265	Palmitelaidic acid	27.14	311	1.5171	3.51E-23	Decreased
11	CHEBI:17351	Linoleic acid	28.21	95	1.5967	7.14E-38	Increased
12	CHEBI:16196	Oleic acid	28.23	96	1.4572	1.74E-38	Increased
13	CHEBI:28842	Stearic acid	28.69	298	1.7235	2.34E-57	Decreased
14	CHEBI:32265	Heptadecanoic acid	28.99	327	1.0286	9.01E-35	Decreased
15	CHEBI:15843	Arachidonic acid	30.51	91	1.4786	3.87E-34	Increased

Table 5: Differential metabolites in male mices between TiO_2 NP exposed and control groups.

S.No	CheBI ID	Name of metabolite	RT (min)	*m/z*	VIP score	*p*- value	Content variance
1	CHEBI:28358	Lactic acid	8.46	190	1.2567	5.22E-31	Increased
2	CHEBI:20067	3-Hydroxybutyric acid	10.65	130	1.4375	3.84E-39	Decreased
3	CHEBI:15428	Glycine	13.98	174	1.2489	4.23E-24	Increased
4	CHEBI:18012	Fumaric acid	14.76	245	1.4406	3.38E-37	Increased
5	CHEBI:17822	Serine	15.24	204	1.4898	7.98E-23	Decreased
6	CHEBI:22660	Aspartic acid	16.50	168	1.285	6.53E-34	Decreased
7	CHEBI:6650	Malic acid	17.92	245	1.0865	6.20E-31	Increased
8	CHEBI: 18183	Pyroglutamic acid	18.47	156	1.7314	8.08E-46	Decreased
9	CHEBI:30915	Ketoglutaric acid	19.62	288	1.5741	1.22E-30	Decreased
10	CHEMSPIDER: 10709816	Phosphopyruvate	19.91	211	2.0992	4.90E-38	Increased
11	CHEBI:26078	Phosphoric acid	22.62	445	1.0075	6.59E-18	Increased
12	CHEBI:18257	Ornithine	22.81	174	2.1934	2.52E-40	Increased
13	CHEBI:30887	Isocitric acid	24.21	465	1.5379	7.77E-36	Increased
14	CHEBI:28875	Tetradecanoic acid	24.30	285	1.7954	1.52E-47	Increased
15	CHEBI:59265	Palmitelaidic acid	25.05	179	1.4911	1.75E-30	Decreased
16	CHEBI:33198	Gluconic acid	27.14	311	1.2888	8.59E-28	Decreased
17	HMDB41480	Cis-13-octadecenoic acid	30.04	339	1.6197	4.41E-27	Decreased

values of PLS-DA model with R^2X_{cum} = 0.474, R^2Y_{cum} = 0.674, Q^2X_{cum} = 0.388, Q^2Y_{cum} = 0.599 shows good fit of the model in male mice (Figure 4B). To assess the statistical significance of these apparently highly predictive multivariate models, permutation testing was conducted. The supervised models were further validated with 1000 times permutation tests ($p<0.001$) (Figure 4C).

Among all the differential variables selected according to the VIP values from the PLS-DA model (VIP score greater than 1), 15 metabolites in female and 17 metabolites in male mice were found in treated group respectively showed in Table 4 and Table 5. In present study, glucose levels were down regulated in serum of treated groups of TiO_2 NP in comparison to control. This down regulation in the level of glucose suggested being as results of increase in the energy requirements of mice exposed to TiO_2 NP. These results showed that disturbance of energy metabolism after exposure to TiO_2 NP [30].

We found, lactic, citric, malic and fumaric acids were significantly ($p<0.05$) increased in serum after exposed to TiO_2 NPs in comparison to control group. Lactic acid acts as a precursor in Krebs cycle (TCA cycle). Citric, malic and fumaric acids were said to be key products in TCA cycle. Citric and lactic acids were closely related to glycolysis and

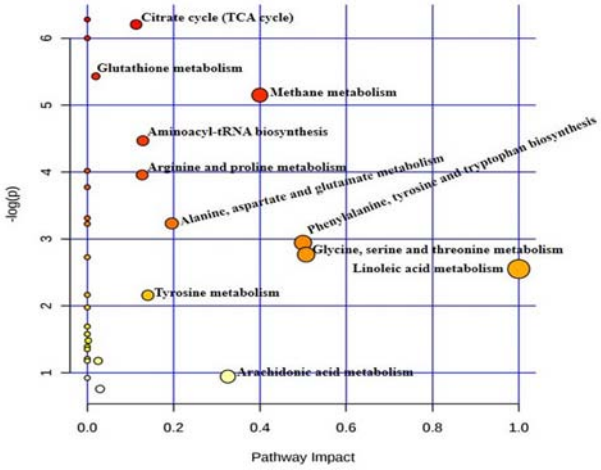

Figure 5: Summary of the pathway analysis of mice exposed to TiO_2NP with MetPa.

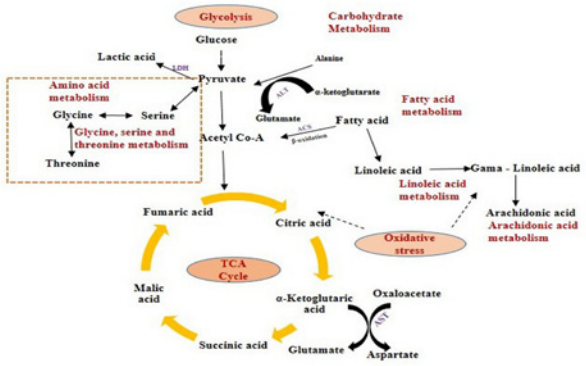

Figure 6: Schematic representation of the affected pathways in Swiss Albino mice due to TiO_2 NP exposure.

energy metabolic pathway in aerobic condition (Figure 6) [31]. The abnormality of citric and lactic acids may be due to induced oxidative stress in the mice due to TiO_2 NPs, which indirectly causes metabolic perturbations in energy metabolism through glycolysis process.

In the study, the level of 3-hydroxybutyric acid was significantly ($p<0.05$) decreased in exposed mice after treatment of TiO_2 NP. Our findings were supported by previous study where, 3-hydroxybutyric acid said to be an end product of fatty acid beta-oxidation pathway produced mainly in the liver and used as an alternative energy source during tissue damage.

The levels of tyrosine and glycine were significantly increased and the levels of serine and aspartic were significantly decreased in treated mice in comparison to control. Glycine is derived from serine with the help of serine hydroxymethyl transferase enzyme and it acts as a cytoprotective agent by scavenging the reactive oxygen species (ROS) and inhibits inflammatory response due to oxidative stress induced by the NPs. Increased serine levels in serum have been related to cellular proliferation and it may decline the cell injury caused by the reactive oxygen species (ROS).

The affected biological pathways in mice after exposure to TiO_2 NP were obtained by using MetPa, a free web-based tool [32]. MetPa reveals importantly, 14 metabolic pathways in mice which showed in Table 6 (Figure 5). Schematic representation of the affected pathways in Swiss Albino mice due to TiO_2 NP exposure showed in Figure 6. Fatty acids are important source of energy. In this study, tetradecanoic, arachidonic, oleic and linoleic acids were significantly increased while, palmitelaidic, stearic, heptadecanoic and cis-13-octadecenoic acids were significantly decreased in serum after exposed to TiO_2 NP. MetPA results showed two pathways related to fatty acid metabolism such as linoleic acid metabolism and arachidonic acid metabolism was significantly affected in treated mice. GC-MS based metabolomic approach can be used as a potential analytical tool to understand the systemic toxicity of NPs in model organisms [33-35].

Conclusion

In conclusion, this is the first comparative metabolomics study on both male and female mice using GC-MS based metabolomics approach to identify the perturbations in metabolic profile after exposed to TiO_2NP. Result of multivariate pattern recognition analysis showed clear molecular group responses to TiO_2NP at metabolic level. The study revealed that the metabolic perturbations occurred in TiO2 NP exposed mice are both concentration and time dependent. The integrated data set indicated clear indication of oxidative stress

Table 6: Affected pathways, metabolites hits and their impacts identified by MetPa in mice.

S.No	Pathway	Metabolite hits	Impact
1	Citrate cycle (TCA cycle)	3	0.1134
2	Glutathione metabolism	3	0.02004
3	Methane metabolism	2	0.4
4	Aminoacyl-tRNA biosynthesis	4	0.12903
5	Arginine and proline metabolism	3	0.12736
6	Alanine, aspartate and glutamate metabolism	2	0.1962
7	Phenylalanine, tyrosine and tryptophan biosynthesis	1	0.5
8	Glycine, serine and threonine metabolism	2	0.50732
9	Linoleic acid metabolism	1	1
10	Tyrosine metabolism	2	0.14045
11	Starch and sucrose metabolism	1	0.00233
12	Cysteine and methionine metabolism	1	0.02533
13	Arachidonic acid metabolism	1	0.32601
14	Primary bile acid biosynthesis	1	0.02976

and alteration of energy metabolism as a result of TiO_2NP exposure. Moreover, our results support the fact that metabolomics approach is a more sensitive than serum biochemistry and haematology analysis. These results showed that metabolomics have a great potential to emerge as a powerful tool to comprehensively understand the toxicity of NPs in model organisms.

Acknowledgment

The authors are thankful to Directors of CSIR-IITR, Lucknow and CSIR-IICT, Hyderabad for scientific discussions and providing the necessary infrastructural facilities to carry out this work. The author(s) received financial support from SERB, New Delhi and CSIR, New Delhi through fast track scheme and NanoSHE (BSC0112) network projects to carry out this research work.

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Austin J Anal Pharm Chem - Volume 4 Issue 2 - 2017
ISSN : 2381-8913 | www.austinpublishinggroup.com

Citation: Singh AK, Ratnasekhar Ch, Chaudhari BP, Singh D, Chattopadhyay BD, Mudiam MKR. Evaluating Metabolic Responses in Mice to Nanosized Titanium Dioxide Particles Using Gas Chromatography-Mass Spectrometry Based Metabolomics. Austin J Anal Pharm Chem. 2017; 4(2): 1085.