NMR based metabonomic approach to understanding metabolic regulatory variation due to acute cold stress

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Introduction: Exposure to acute stressful conditions is responsible for affecting multiple biochemical regulatory systems & triggering various disorders. Acute stress such as extreme cold or heat, panic, toxins, tension etc may result in development of neuro-psychiatric symptoms such as depression, cognitive impairment, and anxiety[1]. Evidence exist for the adverse effects of acute cold stress on human health including cardiovascular & respiratory diseases like hypertension & asthma, diseases relating to immune system, diarrhea etc[2]. The emerging metabolite profiling method, using NMR in conjugation with computer based data reduction & pattern recognition methods such as Principle Component Analysis (PCA) can be successfully used to capture biochemical changes in psychologically-stressed rats.

Aims & Objectives: To study the changes in metabolic profiles of urine in rats due to acute cold stress using ^{1}H high resolution NMR spectroscopy.

Materials & Methods: 8-10 week old male Sprague Dawley rats (200±20g) were housed individually in stainless steel wire mesh cages. Room temperature & humidity were regulated at 24±1ºC & 40±10%, respectively. Rats were acclimatized in metabolic cages & they were divided into two groups, control (C, n= 6) & cold exposure group (CE, n= 6). On day 1, rats of CE group were exposed to -10ºC for 3hrs. Urine samples were collected for each animal in C & CE group on day 1, 4, 5 & 10. To remove particle contaminants, all urine samples were centrifuges at 8000 rpm for 15mins. For NMR analysis, samples (300µL) were prepared by mixing 300µL of D_2O as a field frequency lock with 1mM TSP (in capillary) as an external reference for spectral acquisition. ^{1}H NMR spectra were acquired on each sample at 400.13 MHz on a Bruker Avance 400 spectrometer at 298K. Water suppression was achieved using 1D NOESY pulse sequence. For each sample, 64 transients were collected into 32K data points with a relaxation delay of 2 s, flip angle of 90° and a mixing period of 100 ms. Concentration for each metabolite was calculated by identifying the peaks & integrating wrt TSP. Multivariate analysis was used i.e. Principle Component Analysis (PCA) to carry out data reduction & by using one way ANOVA, the bin values showing significant difference ($P< 0.05$) were calculated to differentiate control from cold stress group.

Results & Discussion: Due to cold exposure of -10ºC for 3hrs, ^{1}H NMR spectra of urine samples (Fig 1) showed statistically significant decrease in metabolites concentration indicating altered biochemical pathways. There was a marked decrease in pyruvate, citrate, 2-oxoglutrate, succinate & fumarate which are key metabolites of TCA cycle indicating up regulation of TCA cycle due to increased energy consumption to provide metabolic regulatory & compensatory mechanism in response to cold stress exposure. This is also supported by decrease in nicotinamide levels as it is important precursor of coenzymes NADH & NADPH which are electron transporters in TCA cycle[3]. Reduced β-hydroxy butyrate levels was observed in response to cold stress. Urinary excretion levels of Creatinine & Hippurate were reduced indicating reduced glomerular filtration rate or alteration in gut microbiota &/ or modifications of transport mechanism at tubular level which maybe related to altered cellular function or low glucose in tublar lumen. Reduced hippurate levels indicate reduced ability of kidney to eliminate acids & maybe considered an early marker for impaired renal function. Decrease in aromatic (tyrosine & phenylalanine) & Trimethylamine-N-oxide (TMAO which is an osmolyte molecule synthesized in renal medulla cells) levels also supports kidney dysfunction[4,5]. Altered phenylalanine & tyrosine levels can also be correlated to enhanced release of catecholamine hormone by adrenal glands in response to stress as phenylalanine is converted to tyrosine by phenylalanine hydroxylase & then to catecholamine (Table 1). This may result in short term functional changes to stress response such as enhanced SNS activity, tensed muscles & rapid heart rate. A 2-D PCA analysis (Fig 2) further strengthened the result & suggested a clear separation between the urine profiles of rats exposed to cold stress in comparison to control group suggesting that exposure to cold stress may lead to systemic metabolic variation.

Metabolites	Control	Cold stress (3hrs at -10ºC)
pyruvate	1.77±0.79	0.75±0.52 *
citrate	40.81±14.19	18.62±6.46 *
2-oxoglutrate	8.76±4.25	3.27±0.99 *
succinate	9.13±4.67	4.04±1.08 *
fumarate	1.39±0.34	0.15±0.09 *
N-methylnicotinamide	3.46±1.05	1.04±0.12 *
creatinine	12.11±2.98	2.99±1.06 *
hippurate	6.77±1.64	2.14±0.85 *
tyrosine	9.88±2.89	2.90±0.96
phenylalanine	7.60±1.63	2.59±1.62 *
β-hydroxy butyrate	6.75±2.85	1.86±1.30 *
TMAO	4.10±1.11	1.19±0.51 *
N-isovaleryl glycine	6.18±2.00	1.93±0.92 *
Alanine	2.07±0.88	0.69±0.52
acetoacetate	4.13±1.35	0.63±0.38 *
glycolate	8.02±2.05	2.17±1.16
alantoin	25.02±7.84	10.48±3.74 *
Formate	0.67±0.27	0.40±0.22

*Significant at 0.05 level

Table 1: The concentration of various metabolites (mmol/µL) for urine samples obtained from control & cold stress exposed rats

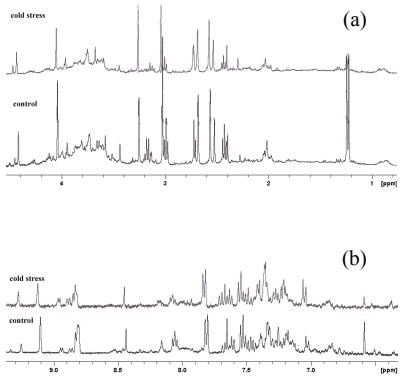

Fig 1: Comparison of expanded ^{1}H NMR spectra of C & CE group urine samples showing decreased intensity of metabolite resonances in CE group. (a) expanded region from δ0.5-5.0 ppm (b) δ6.0-10.0 ppm.

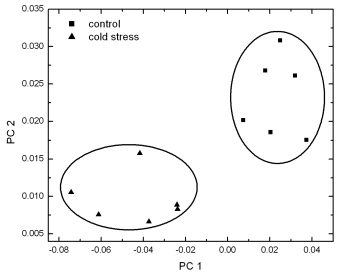

Fig 2: PCA plots from urine samples showing distinct variation between control & cold stress group of rats

Conclusion: Noninvasive monitoring of various biochemical pathways can be done by studying the urinary metabolite profile using NMR spectroscopy in conjugation with multivariate statistical techniques. These preliminary studies will be further used in carrying out risk assessment & developing methods to combat variable climatic conditions.

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