Studies on Metabolic Alterations due to Chronic Cold Stress: An NMR based Metabonomics Apporach

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Introduction: Stressors have been shown to cause an array of endocrine, cardiovascular, and behavioral responses indicative of increased anxiety and fear-related behaviors[1]. Stress is a normal component of everyday life, yet chronic stress has been linked to the emergence and exacerbation of psychiatric disorders[2]. In severe stressful conditions like cold and hypoxia, higher rate of metabolism is required to cope with increased energy demand[3,4]. Cold stress results in increased sympathetic activity, immune suppression, gastrointestinal mucosal damage, affects central nervous system (CNS), hypovolemia, negative electrolyte balance & potential dysfunction to adrenal glands, kidney & lungs. These changes in metabolic activities of cells/tissues can directly be reflected in biological fluids & can be studied using NMR spectroscopy or LC-MS.

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

Material & Methods: 8-10 week old male Sprague Dawley rats (230±30g) 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 0, CE group was exposed to 4ºC for 8 hrs (9a.m. to 5p.m.). Continuous exposure was given to CE1 for 10 & 15 days & urine samples were collected from C & CE groups at different time point, respectively. Thereafter, CE group was brought back to room temperature & urine samples were collected on day 2 & day 10 to study recovery process. To remove particle contaminants, all urine samples were centrifuged at 8000 rpm for 15mins. For NMR analysis, samples 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. One way ANOVA was used to calculate significant difference between metabolite concentrations ($P< 0.05$) & differentiate control from cold stress & recovery phase.

Results: ^{1}H NMR spectra of urine samples for cold exposed rats showed a marked continuous increase in metabolites viz 2-oxoglutrate, citrate, acetoacetate, glycolate, creatinine, fumarate & N-methylnicotinamide after 10 days exposure. Further exposure upto 15 days showed significant increase in 2-oxoglutrate & citrate concentration where rest of the metabolites remained almost unchanged indicating acclimatization. β-hydroxy butyrate, alanine, pyruvate & formate showed an increase & then decrease in metabolite concentration after 10 & 15 days exposure, respectively. Recovery phase on day 2 & day 10 post exposures showed further decrease in metabolite concentration which was comparable to controls. This was further confirmed by ANOVA which showed no significant difference between metabolite concentrations for control & recovery phase for CE group (Table 1).

Discussion: Visual comparison of ^{1}H NMR spectra (Fig. 1) of urine samples between control and cold exposure for 10 days (8hrs each day) showed statistically significant increase in energy metabolites such as 2-oxoglutrate, citrate, fumarate & N-methylnicotinamide indicating perturbation in biochemical pathways. Exposure up to 15 days showed further significant increase in citrate & 2-oxoglutrate concentration whereas other metabolites did not show any significant changes indicating acclimatization to cold exposure & inhibition of TCA cycle. Exposure to chronic cold stress results in a transient lack of ATP which favours direct oxidation of glucose after reaching certain concentration level point. This results in the production of end products of anaerobic metabolism & producing NADPH which is supported by increase & then slight decreases in N methyl nicotinamide levels as it is an important precursor of coenzymes NADH & NADPH which are electron transporters in TCA cycle[5]. Increased alanine also suggests a metabolic switch towards energy conservation. Increase in creatinine concentration due to chronic cold stress indicates altered glomerular filtration rate or alteration in gut microbiota &/ or modifications of transport mechanism at tubular level which may be related to altered cellular function or low glucose in tublar lumen. Significant increase & then decrease in branched amino acid i.e. β-hydroxy butyrate also supports kidney dysfunction[6]. Formate in urine arises due to action of microbes in the gut or as a product of metabolism in the body. Hence, increase in formate indicates disturbed gut flora or increased energy intake. Study of recovery process post chronic cold exposure for 15 days showed an initiation of recovery process with a decrease in metabolite concentrations after 2 days post exposure & further decrease in concentration comparable to controls after 10 days post exposure indicating a complete recovery from cold stress after 10 days of normalization.

Metabolites	Control	Day 10	Day 15	Recovery day 2	Recovery day 10
β-hydroxy butyrate	74.49±59.13	612.14±246.81*	228.45±131.31*	89.52±58.52	45.39±35.93
Alanine	3.74±2.54	7.76±2.39*	5.79±2.74	4.28±1.25	4.77±2.11
Pyruvate	5.91±3.85	11.36±3.71*	8.39±6.72	6.02±2.58	6.23±3.87
2 oxo-glutrate	22.23±19.49	55.40±14.84*	104.89±67.94*	23.78±6.93	25.86±17.43
Citrate	103.79±109.15	228.69±53.41*	263.92±127.89*	95.26±41.51	126.24±78.10
Acetoacetate	8.56±7.04	28.30±6.84*	29.64±21.64*	13.25±5.65	16.37±11.28
Glycolate	19.99±12.67	38.06±8.96*	42.05±25.94	21.0±10.43	28.37±19.32
Creatinine	21.50±18.49	36.27±8.42*	37.34±22.23	22.84±9.27	29.60±13.64
Fumarate	1.19±1.91	7.19±3.49*	6.85±2.92*	2.94±2.09	2.76±2.62
N-methyl nictonamide	7.22±5.32	17.85±6.49*	13.69±7.74	5.28±3.09	8.27±5.12
Formate	2.53±1.86	6.96±2.80*	2.92±1.93	0.88±0.69	0.75±1.15

*significant at 0.05 level

Table 1: The concentration of various metabolites (µmol/g wet weight) for urine samples obtained from control & cold stress exposed rats

Conclusion: NMR based metabonomics serves as a powerful tool for non-invasive monitoring of various metabolic alterations in urine induced by prolonged cold stress. These results can be correlated with other biochemical, analytical & molecular parameters to detect early biomarkers for cold stress injuries in humans which will help in developing strategies to combat variable climatic conditions and mass screening for risk assessment.

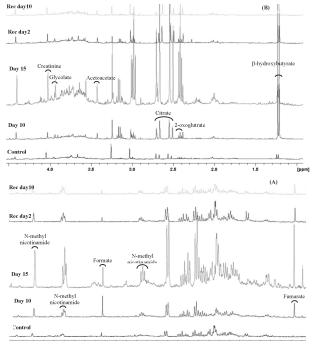

Fig 1: Comparison of ^{1}H NMR spectra of C, CE1 group urine samples showing increased &then decrease in intensity of metabolites with increased duration of cold exposure & recovery phase

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