Urinary metabolic profiling in rats using 1H high resolution NMR spectroscopy to study metabolic alterations due to heat stress exposure

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Introduction: Thermal condition influences the development of living organisms in a wide variety of ways triggering various adaptive responses. Exposure to heat stress can serve as a significant problem as it affects physiological & cognitive performance in humans[1], alter the concentration of selected neurotransmitters & hormones[2], affects the development of neural pathways & hippocampal activity in rats[3], causes hypohydration which affects cognitive performance & effects on gene expression[4,5]. NMR spectroscopy serves as a powerful noninvasive tool to identify biomarkers reflecting the early biochemical changes due to heat stress. NMR based urinary metabolic profiling studies after exposure to heat stress are almost negligible in literature & these studies in conjugation with statistical analysis can be used to detect variation between groups due to stress exposure.

Aim & Objective: To access the changes in metabolite pattern & identify potential biomarkers in rat urine samples due to exposure to heat stress.

Material & 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) & heat stress group (H, n=6). The animal room was maintained on a 12h light/ 12h dark cycle. On day1, rats of H group were exposed to 45ºC±3ºC for 2hrs. Urine samples were collected for each animal in C & H group on day 1, 5 & 10. To remove particle contaminants, all urine samples were centrifuged 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 heat stress group.

Results: The ^{1}H NMR spectra of urine samples for heat exposed rats (Fig 1) showed a marked decrease in metabolites viz pyruvate, citrate, creatinine, phenylalanine & hippurate whereas opposite trend was observed in formate levels. However, rest of the metabolites seen in the ^{1}H NMR spectra did not show any significant difference between control & heat stress rats (Table 1).

Discussion: Exposure to heat stress at 45ºC±3ºC for 2hrs resulted in metabolic changes causing a reduced level of phenylalanine which can be correlated to, increased release of catecholamine hormone by adrenal glands which can result in increased heart rate & fatigue with onset of thermoregulatory mechanism. This may cause increased skin blood flow reducing cardiac return in an attempt to move more blood to periphery to transfer heat out of the body by convection[7]. There are evidences indicating morphological changes in adrenal glands of rats when exposed to heat stress resulting in increased plasma corticotrophin & serum corticosterone concentration[8]. Creatinine & hippurate concentration was reduced indicating altered renal function &/ or gut microbiota. This can serve as an early marker as it indicates reduced ability of kidney to eliminate acids. There was down regulation in pyruvate & citrate concentration indicating increase TCA cycle activity. Irrespective of any kind of physiological stress, there is increased energy consumption & protection against internal & external stress is provided by allostatis (ability to achieve stability through change). Reverse trend was observed for formate concentration. 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. PCA plot (Fig 2) showed clear separation between control & heat stress group.

*Significant at 0.05 level

Metabolites	Control	Heat stress (2hrs at 45ºC)
N-isovaleryl glycine	5.41±1.17	3.14±2.16
β-hydroxy butyrate	6.49±3.23	7.37±9.85
Alanine	1.83±0.81	1.09±0.61
N-acetylglycoprotein	8.90±2.24	6.55±4.53
pyruvate	1.80±0.91	1.23±1.40 *
succinate	7.59±3.63	5.48±2.52
2-oxoglutrate	7.49±3.67	4.44±2.89
citrate	35.63±9.50	19.54±13.37 *
creatinine	8.99±1.92	6.97±5.99 *
malonate	2.57±1.18	1.80±0.94
cis-aconitate	4.22±2.80	1.41±1.17
TMAO	3.64±0.46	2.29±1.52
acetoacetate	3.91±1.45	3.23±3.22
glycolate	7.33±1.57	5.19±5.57
N-methylnicotinamide	3.18±0.99	2.38±2.05
alantoin	22.0±4.60	18.74±15.42
fumarate	1.34±0.38	0.70±1.05
phenylalanine	7.55±1.88	4.13±3.85 *
hippurate	6.22±1.24	2.90±2.22 *
Formate	0.60±0.25	1.37±1.74 *

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

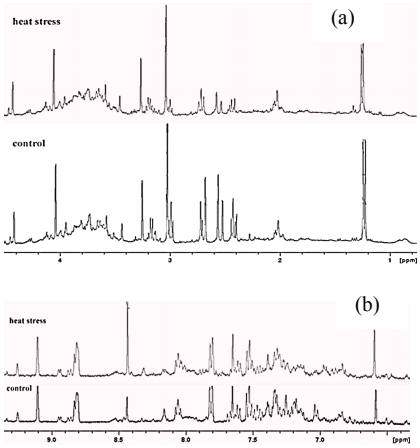

Fig 1: Comparison of expanded ^{1}H NMR spectra of C & H group urine samples showing decreased intensity of metabolite resonances in H group. (a) expanded region from δ0.5-5.0 ppm (b) δ6.0-10.0 ppm.

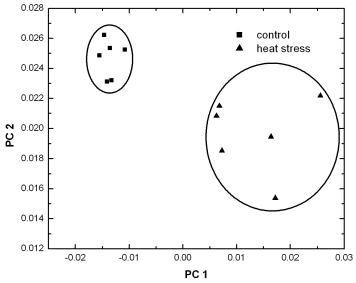

Fig 2: PCA plots from urine samples showing distinct variation between control & heat stress group of rats

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Conclusion: NMR based metabonomic studies in conjugation with statistical analysis permits noninvasive & simultaneous monitoring of entire metabolic pathways. This reveals the subtle interplay of functional metabolites & pathways leading to an understanding of the systemic response to external stimuli such as heat stress. This can help in developing strategies to sustain heat stress.