Comprehensive analysis of urinary metabolites from sprague-dawley (SD) rats when exposed to acute heat stress

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Abstract

Acute heat stress may trigger systemic biochemical and physiological changes in living organisms leading to rapid loss of homeostasis. Metabolic regulatory process during heat stress has been poorly understood therefore the present study was undertaken in order to have an insight of alterations in various metabolites due to acute heat stress exposure using nuclear magnetic resonance (NMR) spectroscopy. Male sprague- dawley (SD) rats were exposed to acute heat stress of 45±2°C for 3 h and urine samples were collected from control (n=6) and heat stress group (n=6) on day 0 and 1. Metabolites excreted in urine were analyzed using NMR spectroscopy in conjugation with principal component analysis (PCA). The biochemical response to acute heat stress was characterized by decreased levels of citrate, succinate, 2-oxoglutrate, phenylalanine, creatinine, hippurate and elevation in formate levels. Hence, some biological pathways such as tricarboxylic acid (TCA) cycle, catecholamine activity and gut microbiota were transiently affected due to heat stress exposure. NMR based metabonomic studies in conjugation with statistical analysis permits non-invasive and simultaneous monitoring of entire metabolic pathways. This reveals the subtle interplay of functional metabolites and pathways leading to an understanding of the systemic response to external stimuli such as heat stress. These studies form the basis for future studies to detect early biomarkers for heat stress in humans and identifying the population at risk. Furthermore, it can be used to develop methods to provide protection to the body against environmental insult, thereby reducing the adverse response to heat stress.

Keywords: Heat stress, hyperthermia, nuclear magnetic resonance, spectroscopy, metabonomics, urine.

INTRODUCTION

Thermal condition influences the development of living organisms in a wide variety of ways triggering various adaptive responses (Gluckman et al., 2005). Exposure to thermal stress poses a significant problem as it affects physiological and cognitive performance in humans (Riniolo and Schmidt, 2006), alter the concentration of

Abbreviations: NMR, Nuclear magnetic resonance; **PCA,** principal component analysis; **SD,** Sprague-Dawley; **TCA,** Tricarboxylic Acid cycle; · **TSP,** 3 -(trimethylsilyl) propionic-2, 2, 3, 3-d4 acid sodium salt; **GIT,** gastrointestinal tract; **SNS,** sympathetic nervous system.

selected neurotransmitters and hormones, affects the development of neural pathways (Horwitz et al., 1982) and hippocampal activities in rats (Karlsoon and Blumberg, 2004), causes hypohydration (Grandjean and Grandjean, 2007; Lieberman et al., 2007), and effects on gene expression (Sonna et al., 2002). Studies have shown a significant decrease in the adrenal ascorbate concentration accompanied by its increase in circulation when exposed to extreme environmental temperature (Djordjevic et al., 2006; Hume and Egdahl, 1959).

Human beings have evolved effective mechanism for dissipating excess heat through the ability of increasing skin blood flow and sweat rate (Rowell, 1983; Nadel, 1985). However, excessive heat stress can exceed the ability of the body to regulate its temperature leading to various heat related illness such as impaired working

efficiency, cause haemoconcentration leading to coronary and cerebral thrombosis, particularly in elderly people with atheromatous arteries (Donaldson et al., 2003) . All these side effects make it necessary to understand the cause of these alterations at metabolic level. Urinary metabonomic study has proven to be a noninvasive technique that can be used to gather samples and data simultaneously from numerous targets thereby overcoming various limitations of other current research methods (Hodson et al., 2007; Ebbels et al., 2004).

The emerging metabolite profiling method, using nuclear magnetic resonance (NMR) and gas chromatography/mass spectroscopy (GC/MS) in conjugation with modern multivariate statistical technique has been successfully used to study biochemical changes in psychologically-stressed rats (Teague et al., 2007). ^{1}H NMR based metabolic profiling of biological fluids, cells and tissue extracts have yielded valuable information by successfully assessing drug safety, diagnosing the presence and severity of coronary heart disease, characterizing early and chronic psychological stress-induced variance and diet intervention, and exploring the intricate relationship between mammalian systems and gut microbiota (Wang et al., 2006; Brindle et al., 2002; Waters et al., 2006).

With ^{1}H NMR spectroscopy of biofluids, the simultaneous measurement of a variety of low-molecular weight metabolites from a range of intermediate metabolic pathways can be done (Nicholson et al., 2003; Sands et al., 2009; Robertson et al., 2005; Nicholls et al., 2003). High-resolution ^{1}H NMR spectra of biological samples are extremely complex, comprising thousands of signals from various metabolites, many of which overlap. Computer based data reduction and pattern recognition methods such as principal component analysis (PCA) have proven to be a very beneficial techniques to make optimal use of the information present in the ^{1}H NMR spectrum for classification purpose (Holmes et al., 2000).

The aim of this study was to provide a comprehensive analysis of urinary metabolites from sprague-dawley (SD) rats when exposed to acute heat stress giving an insight of induced biochemical responses and metabolic consequences.

MATERIALS AND METHODS

Animal handling and sample collection

Eight-week-old male sprague-dawley rats (for stable metabolic status) (200±20g) were obtained from experimental animal facility of the institute and were housed individually in the cages, and fed with a certified standard rat chow and tap water *A. libitum*. All the animal studies and handling were conducted in accordance with institutional animal ethical committee guidelines (Reg. No. 8/1999/CPCSEA). Room temperature and humidity were regulated at 25±2°C and 40±15%, respectively. A light cycle of 12 h light and 12 h dark was set with lights on at 8.30 am. After 1 week of acclimatization in metabolic cages, randomly rats were divided into two groups namely; control (C, n=6) and heat stress group (HS,

n=6). Baseline urine sample was collected from each animal on day 0 before heat exposure. On day 1, HS group rats were exposed to 45±2°C heat stress for 3 h in an in-house fabricated chamber. Rats were supplied with rat chow and tap water in the chamber to prevent dehydration. Rats were then taken out and returned to metabolic cages at room temperature under normal conditions without disturbances.

12 h urine samples were collected (Wang et al., 2007) in vials containing 1 mL of 0.1% sodium azide, from each animal of C and HS group at day 0 and 1. Particle contaminants were removed by centrifuging the urine samples at 8000 rpm for 10 min and the resultant supernatants were stored at -80°C for NMR analysis.

Sample preparation and ^{1}H NMR spectral acquisition

300µL of urine sample was mixed with 300 µL of buffered deuterium oxide (0.2M Na_2HPO_4/0.2M NaH_2PO_4, pH7.0, D_2O, Aldrich, 99.9%) and transferred to 5mm NMR tubes containing 1mM 3-(trimethylsilyl) propionic-2, 2, 3, 3-d4 acid sodium salt (TSP) solution as an external standard for spectral acquisition. ^{1}H NMR spectra were acquired on each sample at 400.13 MHz on a Bruker Avance 400 spectrometer at a probe temperature of 298K. Water suppression was achieved using 1D NOESYPR pulse sequence. A standard one-dimensional water peak pre-saturation pulse sequence (90°-t_1 -90°-t_m-90°-ACQ) was applied. The interpulse delay t_1 was 3 µs, and the mixing time t_m was 100ms.

A weak irradiation field was applied at the water resonance frequency during both the mixing time and the recycle delay. For each sample, 64 transients were collected into 32K data points with a relaxation delay of 2 s and flip angle of 90°. Concentration for each metabolite was calculated by identifying the peaks, integrating with respect to TSP and using these integral values in the following equation:

$$[C]_X = [C]_{TSP} N_{TSP} . I_X / N_X . I_{TSP}$$

Where, $[C]_X$ is the concentration of metabolite X, I_X and I_{TSP} are the NMR signal intensities of X and TSP, respectively. N_X is the number of protons per molecule giving rise to the integrated signal and N_{TSP} = 9. Metabolite ratios were calculated from the integrated intensity of individual peaks (Sharma et al., 2001).

Spectral processing, data reduction and pattern recognition

For all 1D ^{1}H NMR spectra, free induction decays were multiplied by an exponential function corresponding to a 0.3Hz line broadening prior to "Fourier transformation", were phased and baseline-corrected using TOPSPIN (Bruker, Germany) . The spectra were referenced to the TSP resonance at 0 ppm. The spectra were reduced to 250 integrated regions of equal width (0.04 ppm) corresponding to the region 0 to 10. Data reduction in this way can diminish the effects of chemical shifts that can exert a relatively strong influence in the data sets where number of samples per group is small. The region 4.6 to 5.5 was removed to avoid residual spectral effects of imperfect water suppression.

Normalization of the spectra to a constant sum was carried out on these data and principal component and statistical analysis (PCA) was done using in house developed algorithm on MATLAB 7.0 (Mathworks, USA). The data obtained was visualized in the form of the principal component (PC) scores plots and loadings plots (Holmes et al., 1998; Robertson et al., 2005) . Scores plots of the PC were constructed to visualize any inherent clustering of the samples between control and heat stress group, and from the value of the PC loadings and NMR spectral regions, changes in metabolites under heat stress condition were identified. Each co-ordinate on the scores plot represents an individual sample and

each co-ordinate on the loading plots represents one NMR spectral region. Thus the loadings plots provide information on spectral regions responsible for the position of co-ordinates or clusters of samples in the corresponding scores plots (Wold et al., 1987).

In addition to this, classical one way analysis of variance (ANOVA) was also utilized to judge whether the results were statistically significant. In this study, the critical p-value was set to 0.05.

RESULTS

^{1}H NMR analysis of rat urine following acute heat stress

Acute heat stress induced perturbations on ^{1}H NMR spectra, showing the alterations in low molecular weight metabolite profiles in response to stress. Figure 1 shows the representative 1D 400 MHz ^{1}H NMR spectra of rat urine for the control (C) and heat stress (HS) group. Comparison between the spectra (with a constant y scale) clearly indicates change in the intensity of the resonance peaks of the metabolites for samples from HS group compared to C group indicating substantial alterations in urine metabolite profile consistent with a perturbation of homeostasis. The changes in the concentration of various metabolites for urine samples obtained from C and HS rats are presented in Table 1 in the form of mean ± standard deviation.

Following acute heat stress, a decrease in the resonance peak intensity of succinate, 2-oxoglutrate, citrate, creatinine, hippurate and phenylalanine was observed and an increase in the intensity of formate wasalso evident when visual comparison of the NMR spectra of the urine samples of C and HS group was done. Changes were observed in the concentration of other low molecular weight metabolites but were not significant at 0.05 levels. Since visual analysis of the ^{1}H NMR spectral profiles is a subjective process and inter-animal variation can easily distort interpretation of these data, multivariate data analysis of NMR spectra was performed in order to form a general overview of metabolite patterns of the effects of heat stress.

Principal component analysis (PCA) of ^{1}H NMR spectra

Two dimensional PCA plots were generated for ^{1}H NMR urine spectrum to systemically address the metabolic responses to acute heat stress. A total of two components PC1 and PC2 were calculated with 98.3% of the total variances being explained. As expected, no separation between C and HS group was observed in the PCA scores plot on day 0 (Figure 2A) whereas exposure to heat stress on day 1 resulted in a clear separation between C and HS group along PC1 and PC2 axis suggesting that the exposure to acute heat stress may lead to systemic metabolic variation (Figure 2B).

PC loadings versus chemical shift were plotted for C and HS group to compare the regions of the spectra and metabolites responsible for the separations in PC scores plot (Figure 3A and B). The loadings plot suggested that the urine samples obtained from heat stressed rats contained altered concentration of citrate (2.66), succinate (2.42), 2-oxoglutarate (2.98), phenylalanine (3.26, 4.06, 7.30, 7.34), creatinine (3.02, 4.06), hippurate (7.54, 7.82) and formate (8.46) in comparison with control rats (Figure 3A and B).

DISCUSSION

Till date most of the metabonomic studies have been carried out on the profiling of drug toxicity, several disorders particularly those from inborn errors of metabolism and evaluating biomarkers of clinical relevance (Vangala and Tonelli, 2007) . A growing awareness that fast–paced lifestyles can cause physiologic and psychological stress has led to an increasing number of experimental studies on various stress–induced diseases (Teague et al., 2007) . Understanding the effect of acute heat stress can give an insight of metabolic alterations which causes the initiation and development of depressive disorders. This can be further used to explore the pathogenesis of physical and mental diseases (Kanayama et al., 1999; Chrousos, 2000).

Out of all the biological fluids, urine sampling is non-invasive and does not create additional stress such as agitation caused by collecting blood samples to assess metabolic changes. In this study, we utilize this non-invasive method to capture the dynamic systemic metabolic variation due to acute heat stress.

Metabolic response to acute heat stress

From previous literature, exposure to heat stress causes different physiologic reactions resulting in physical stimulus increased skin blood flow (Nielsen, 1993), sweat rate as well as activating the central nervous system (Doris and Baker, 1981). The results of the present study demonstrates the impact of heat exposure for 3 h, on metabolic pathways as indicated by the up or down-regulated levels of low molecular weight metabolites in HS group compared with the C group. The results of the present study showed significant effects on the metabolites involved in several pathways such as tricarboxylic acid (TCA) cycle (citrate, 2-oxoglutrate, succinate) (Michaud et al., 2008; Gibala et al., 1997), gut microbiota metabolic activity (creatinine, hippurate), phenylalanine and formate levels which was in agreement with the previous literature on alterations induced by physiological stress (Bollard et al., 2005; Blomstrand et al., 1992; Janus et al., 2005). Calculation of the concentration of metabolites using integral values from ^{1}H NMR spectra and finding significant changes
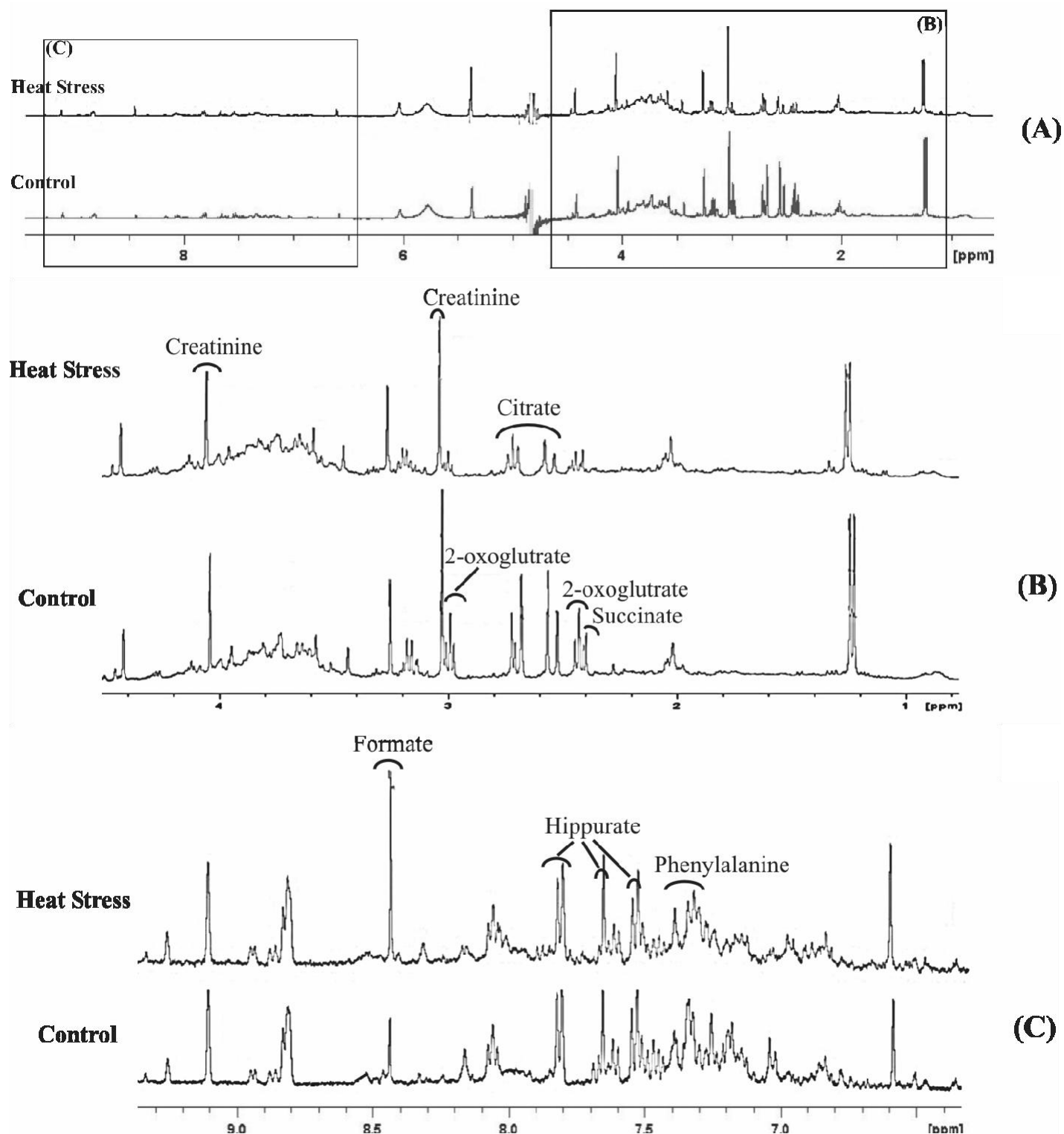
Figure 1. Comparison of expanded ¹H NMR spectra of C and HS group urine samples showing decreased/increased intensity of metabolite resonances in HS group. (A) Complete ¹H NMR spectra of C and HS group, (B) expanded region from 0.5 to 5.0 ppm and (C) 6.0 to 10.0 ppm.

using ANOVA (P < 0.05) indicated that a number of important members of the TCA cycle were altered in rat

Table 1. The concentration of various metabolites (mmol/µL) for urine samples expressed as mean ± standard deviation with significant difference at 0.05 level obtained from control (C) and heat stress (HS) exposed rats.

| Metabolites	Control	Heat stress (3h at 45±2°C)
N-isovaleryl glycine	5.41±1.17	3.14±2.16
-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 *

*Significant at 0.05 level.

urine post heat stress for 3 h. The urinary excretion levels of citrate, 2-oxoglutrate, and succinate were decreased. As reported earlier, irrespective of any kind of physiological stress, there is increased energy consumption and protection against internal and external stress is provided by allostatis (ability to achieve stability through change) (Michaud et al., 2008; Kulinskii et al., 1986; Sterling and Eyer, 1988). During heat exposure, increased energy consumption is expected. However, in our studies the decrease in TCA cycle metabolites can be explained by two stages. Initially, during heat exposure, TCA cycle is accelerated due to enhanced adrenergic nerve activity. As soon as the rats are returned back to metabolic cages, they appeared less active indicating initiation of recovery process and thus slower energy consumption period. The short-term exposure to acute heat stress followed by long room temperature recovery process leads to overall lower level of TCA cycle metabolites in twelve hours urine sample. Hence, alteration of the TCA cycle is an important part of metabolic regulatory and compensatory mechanism in response to heat stress exposure.

From previous literature indicating the effects of different type of stress, it can be said that increased glucocorticoid secretion and enhanced sympathetic nervous system (SNS) activity are two major upstream metabolic regulatory pathways activated due to heat stress exposure (Miller and O'Callaghan, 2002). Studies have shown that exposure to heat stress causes morphological and stereological changes in rat adrenal glands which are the major site for glucocorticoid synthesis (Koko et al., 2004). Under heat stress condition, animals are in hyperthermia (characterized by increased body temperature) which results in salivation, water and salt loss hence to re-establish osmotic homeostasis, increased amount of aldosterone (the salt-retaining hormone) is required. Such metabolic compensatory regulation facilitates protection of living organism from stress-induced damage (Aguilera et al., 1996).

In present study, phenylalanine level, an essential amino acid, decreases on exposure to acute heat stress. Phenylalanine is first converted to tyrosine by phenyl-lanine hydroxylase and then to catecholamine (hormone released by adrenal glands in response to stress) (Blomstrand et al., 1992) . Decreased phenylalanine levels can be attributed to its increased conversion to catecholamines in response to heat stress. These alterations indicate enhanced SNS activity, leading to an up-regulated catecholoamine metabolic pathway (Wang et al., 2009). This may lead to physiologic effects such as increased heart rate, increase in sweat rate and salt loss, increased rectal and body temperature. The results showed a decrease in creatinine levels due to heat stress exposure. Creatinine is a break-down product of creatine
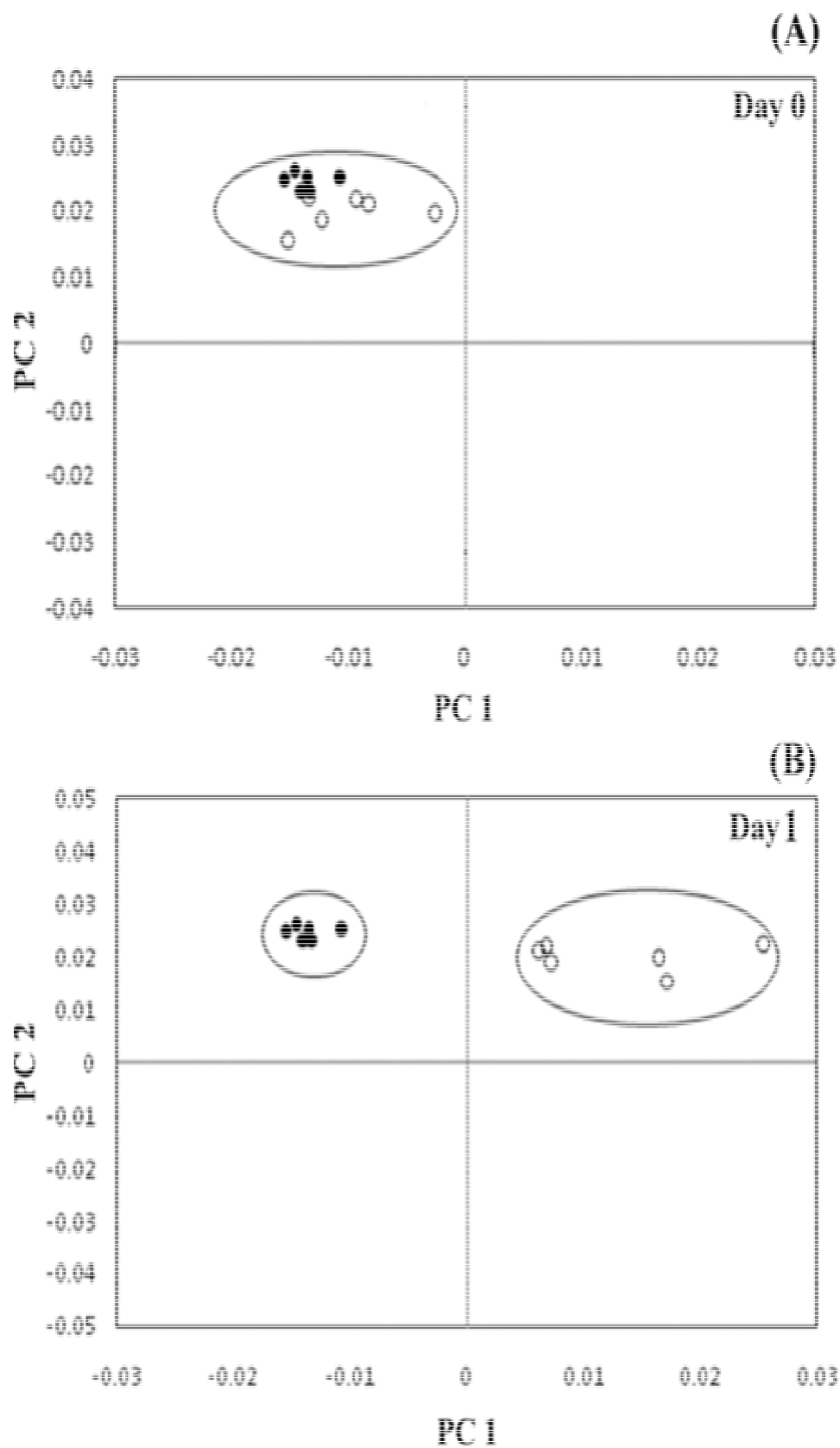
Figure 2. Score plots (PC1 vs PC2) from PCA analysis of ^{1}H NMR spectra of urine from control ( ) and heat stress ( ) group. (A) showing no distinction between groups with no heat exposure on day 0 and (B) separation between groups when exposed to heat stress on day 1.
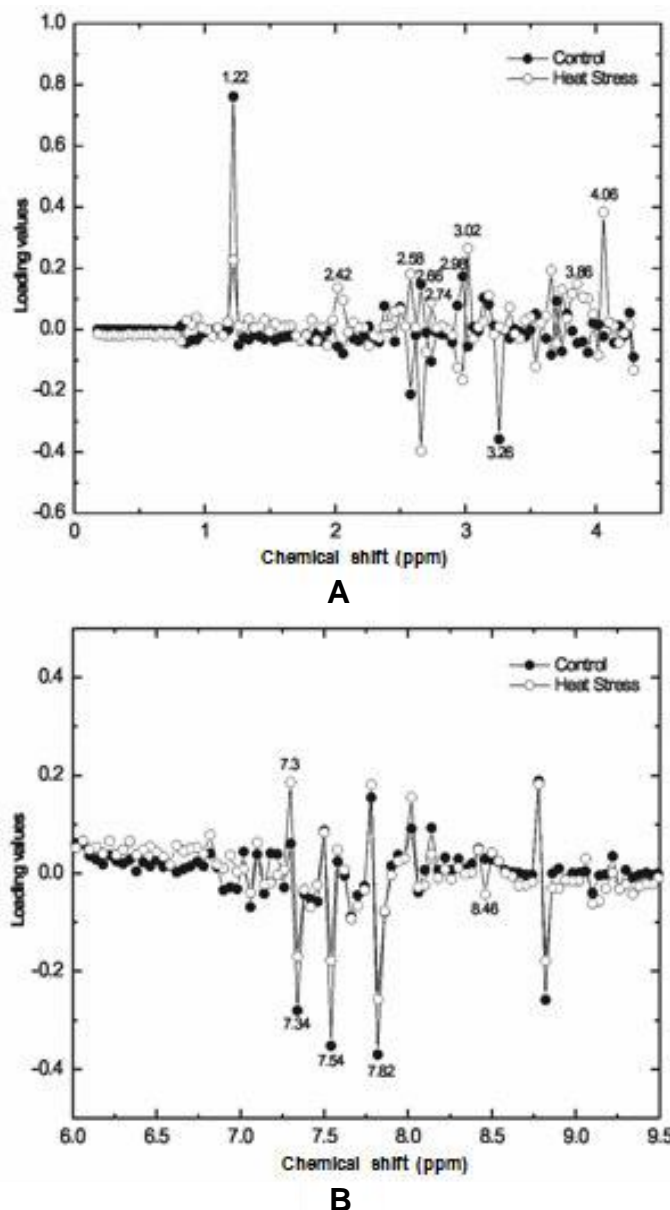
Figure 3. Loading plots from analysis of [1] H NMR spectra of urine samples from controls ( ) and heat stress ( ) groups representing chemical shift positions corresponding to metabolites altered due to heat stress.

phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). Creatinine levels in urine may be used to calculate the creatinine clearance (CrCl), which reflects the glomerular filtration rate (GFR). The GFR is clinically important because it is a measurement of renal function.

Reduced creatinine may be indicative of reduced glomerular filtration rate and/or modifications of transport mechanism at tubular level which may be related to altered cellular function or low glucose in tubular lumen. Reduced creatinine levels might also indicate reduced

ability of kidney to eliminate acids and may be considered as an early marker for impaired renal function. Additionally, our results show decrease in hippurate levels. Hippurate is believed to be metabolized by gut microbial community indicating significant involvement of gut microbiota (Dumas et al., 2006) in response to heat stress. These observed affects on gut microbiota is interlinked with stress-induced variation of catecholamines and noradrenaline as they coexist with gut microflora in gastrointestinal tract (GIT) (Lyte and Bailey, 1997; Hawrelak and Myers, 2004).

There was an increase in formate levels that was observed due to heat stress exposure. The trend observed was opposite as compared to other metabolite changes due to heat stress. 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 (Janus et al., 2005). The results and findings of the present study indicating the metabolic alterations due to acute heat stress has been presented in Joint Annual Meeting ISMRM-ESMRMB 2010 (Gandhi et al., 2010).

Conclusions

NMR based metabonomic studies in conjugation with statistical analysis permits non-invasive and simultaneous monitoring of various metabolic pathways revealing a subtle interplay of functional metabolites and pathways leading to an understanding of the systemic response to external stimuli such as heat stress. Our results provides a new insight into the changes induced by heat stress at metabolic level showing a decrease in metabolites involved in TCA cycle (succinate, 2-oxoglu-trate and citrate) and catecholamine metabolic pathway (phenylalanine). Reduced creatinine and hippurate levels due to heat exposure may be considered as an early marker for impaired renal function and altered gut microflora, respectively. With the help of NMR-statistical analysis, heat stress group can be separated from control group. These studies will be further extended with clinical parameter and histological studies on tissues to form the basis for detecting early biomarkers for heat stress in humans and identifying the population at risk. Additionally, it can be used to develop methods to provide immunity to the body against environmental insult, thereby reducing the adverse response to heat stress.

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