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Nuclear Magnetic Resonance (NMR)-Based Metabolomic Studies on Urine and Serum Biochemical Profiles after Chronic Cysteamine Supplementation in Rats

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ABSTRACT: The purpose of this study was to investigate the effect of chronic cysteamine (CS) supplementation on rat metabolism. Rats received biweekly intragastric administration of either CS−HCl at 250 mg/kg body weight or saline (control) for 4 weeks. The 24 h urine and blood serum samples after the last CS treatment were analyzed by nuclear magnetic resonance (NMR)-based metabolomics, specifically high-resolution ^{1}H NMR metabolic profiling combined with multivariate statistics. Metabolic effects of CS include decreased serum acetate, trimethylamine-*N*-oxide, and urine hippurate, together with increased urine dimethylamine, indicating modulation of intestinal microbial metabolism of the rats. A decrease in urine succinate, citric acid, and serum acetoacetate, together with an increase in serum lactate, was also observed, which suggests that CS supplementation results in perturbation of energy metabolism in rats.

KEYWORDS: Cysteamine, metabolism, metabolomics, NMR spectroscopy

INTRODUCTION

Cysteamine (CS; β-mercaptoethylamine, HS−CH_2−CH_2−NH_2) is biologically derived from cysteine metabolism. As the terminal and active site of the coenzyme A molecule, CS is not only a normal body constituent but occupies a unique position in intermediary metabolism as well. CS is a precursor of hypotaurine, which is subsequently oxidized to taurine. It also participates in the activation of various cholic acids. Plasma lactate and glucose concentrations increase after acute CS administration, peak 2 h after treatment, and decline to pretreatment concentrations by 24 h.[1] Acute CS administration also increases net portal-drained viscera release of β-hydroxybutyrate in sheep,[2] while chronic CS supplementation can regulate growth performance and insulin-like growth factor systems in animals.[3] CS can increase protein deposition by decreasing protein breakdown.[4] Gavage feeding of very high doses of CS to rats reliably produces duodenal ulcers within 24 h.[5] Furthermore, CS has been found effective in protecting animals from an ionizing radiation injury when injected prior to exposure and has antioxidant properties.[6] CS has been applied for many years to treat children with cystinosis successfully.[7] It may also be used as an endogenous regulator of immune system activity and is a potential therapeutic agent for the treatment of Huntington disease.[8] Moreover, it can delay the development of neurological symptoms, reduce apoptosis, and help retain brain volume, thus slowing disease progression.[9]

Traditionally, studies on CS administration have been achieved by measuring and comparing a single or several biochemical marker(s), which does not sufficiently reflect the overall metabolism status of the animals or human. These parameters are usually preselected based on a hypothesis and then verified. However, this approach is limited as a means of unbiased global screening of physiological perturbations, such that detection of unexpected or novel mechanistic phenomena or markers is almost impossible using this approach. Thus, a robust method that can simultaneously quantify and identify a large number (hundreds to thousands) of molecules is necessary. Molecular systems biology has been proposed as an alternative and promising resolution for understanding and elucidating the metabolic mechanisms of all kinds of substances, such as drugs and diet. The development of analytical techniques has enhanced the capability for global assessment of entire classes of biomolecules, such as genome, proteome, and metabolome. Metabolomics provides a useful system approach to understanding global changes of metabolites in animals or human in response to alterations in nutrition, genetics, environments, and gut microbiota.[10−12] This field has become increasingly important in understanding biological processes, and the information obtained from metabolomic studies complement those from proteomics and genomics. At present, metabolic profiles can be more comprehensively characterized using high-throughput analytical tools, such as proton nuclear magnetic resonance (^{1}H NMR) spectroscopy. ^{1}H NMR spectroscopy of biofluids or tissues generates comprehensive biochemical profiles of low-molecular-weight metabolites that are regulated in response to various stimuli to maintain homeostasis. Currently, metabolomics has been applied in various biomedical, toxicological, and nutritional studies, because alterations in the physiological status can disrupt homeostasis and result in perturbations of the levels of endogenous biochemicals involved in different key metabolic processes. Thus, monitoring perturbations of biofluid composition can provide valuable information to understand molecular mechanisms and novel insight into alterations in the physiological status of biological systems. However, few studies have focused on the response of animal or human biological systems to chronic CS supplementation.

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This study implements a ^{1}H NMR-based metabolomic strategy to investigate global metabolic response to chronic CS administration in rats. Metabolic profiles of CS supplementation in rats can improve the current understanding of the relationship between metabolites and CS supplementation and establishes baseline data for future metabolomic experiments on CS metabolism. This approach is also potentially useful to the study of CS metabolism and the search for further associations between CS administration and health or disease risk. Furthermore, this study may help in the determination of biochemical mechanisms involved in cystinosis disease, Huntington disease, and other inflammation-related diseases affected by CS treatment. This study aims to investigate the effect of chronic CS administration on the urine and serum composition of rats using explorative metabolomic analysis through ^{1}H NMR spectroscopy and chemometrics.

MATERIALS AND METHODS

Treatment and Sample Collection. Experimental protocols used in this study were approved by the Sichuan Agricultural University Institutional Animal Care and Use Committee. A total of 22 male Sprague−Dawley rats weighing 240−250 g (approximately 9 weeks old) were placed in individual cages and allowed an initial acclimatization period of 2 weeks. Afterward, the animals were transferred to individual metabolic cages and allowed to acclimatize further for 48 h. Rats received biweekly (Monday and Thursday) intragastric administration of either CS−HCl (Sigma Chemical Co., St. Louis, MO) at 250 mg/kg body weight or saline (control) for 4 weeks. Six rats were selected for each group. Urine samples were collected continuously into ice-cooled vessels at 0−24 h after the last CS administration. Serum samples were also collected (0830) from the orbital sinus into standard heparin-free Vacutainer tubes 24 h after the last post-administration of cysteamine. Blood was centrifugated at 3500*g* for 10 min at 4 °C to obtain serum. All urine and serum samples were stored at −80 °C until NMR spectroscopic analysis. The dosage selected for this study was based on a prior experiment.[13] Free access to feed and drinking water was provided. The temperature between 22 and 25 °C, a cycle of 12 h of light/12 h of dark, and humidity from 50 to 70% were maintained throughout the study. Clinical observations were performed during the experimental period, and body weights were determined once a week. After final sample collection, all animals were killed by decapitation after halothane anesthesia and subjected to autopsy.

Screening of Urine and Serum Samples by ^{1}H NMR Spectroscopy. To minimize gross variations in pH of the urine samples, 350 μL of a buffer solution (0.2 M Na_2HPO_4/NaH_2PO_4 at pH 7.4) was mixed with 350 μL urine in a microcontainer. The resulting solution was left to stand for 10 min and then centrifuged at 13 000 rpm for 10 min to remove precipitates. A 600 μL aliquot of the supernatant was added to 30 μL of 1 mM 3-(trimethylsilyl) propionic-(2,2,3,3-d_4) acid sodium salt (TSP) in D_2O. ^{1}H NMR spectra were acquired on a Varian INOVA 600 spectrometer, operating at a proton frequency of 599.97 MHz at 26 °C using a standard one-dimensional pulse sequence, that is, RD−90°−t_1−90°−t_m−90°−acquire data. Here, the probe (5 mm outer diameter ^{1}H−^{13}C Z gradient) was used for urine sample delivery. Water suppression was achieved with weak irradiation on the water peak during the recycle delay (RD = 2 s). The mixing time, t_m, was set at 150 ms. t_1 was set at 3 μs. A 90° pulse length was set at ∼10 μs. A total of 64 transients were acquired into 32 000 data points using a spectral width of 8000 Hz and an acquisition time of 4 s.

Serum samples were prepared for NMR by dilution with 300 μL of serum 1:1 with D_2O and centrifugation at 13 000 rpm for 10 min. Supernatants (550 μL) were transferred into 5 mm NMR tubes. ^{1}H NMR spectra of the serum were measured using a 5 mm triple-response probe. NMR spectra were measured using the Carr−Purcell−Meiboom−Gill (CPMG) spin−echo pulse sequence to emphasize resonances from low-molecular-weight metabolites. Spectra were obtained with a spin−echo delay τ of 400 μs and a total spin−spin relaxation delay ($2n\tau$) of 320 ms. Other acquisition parameters were the same as described above. Prior to Fourier transformation, an exponential line broadening function of 0.5 Hz was applied to the free induction decay (FID).

Data Processing and Multivariate Analysis. All FID data were Fourier-transformed, manually phased, and baseline-corrected using VNMR software (Varian, Inc.). ^{1}H NMR chemical shifts in urine were referenced to TSP at δ 0.0. The region that contained the resonance from residual water and urea (δ 4.6−6.2) was excluded to eliminate spurious effects of variability in water resonance suppression and the effects of urea signal variations caused by partial cross-solvent saturation via solvent-exchanging protons. After the removal of these redundant regions, each spectrum over the range of δ 0.2−9.4 was segmented into regions 0.04 ppm wide and each spectral intensity data set was normalized to the total sum of the spectral integrals. For serum, NMR spectra were referenced to the lactate−CH_3 signal at 1.33 ppm. Each spectrum over the range of δ 0.4−4.4 was data-reduced into integrated regions of equal width (0.01 ppm). The integral of each region was determined. Resultant data sets were then imported into Microsoft Excel, version 2003. Metabolite assignments were usually made by consideration of the chemical shifts, coupling constants, and relative intensities as in previous reports[14,15] and additional two-dimensional NMR experiments on selected samples.

Multivariate data analysis, including principal component analysis (PCA), projection to latent structures (PLS), and orthogonal signal correction (OSC), were performed with the SIMCA-P 10.04 (Umetrics AB, Umeå, Sweden) software package. All variables were preprocessed with mean-centered and Pareto-scaled prior to variable analysis. Each principal component (PC) is a linear combination of the original variables, with each successive PC explaining the maximum amount of variance possible in the data set and with each PC being orthogonal to every other PC. Data were visualized by plotting the PC scores and PC loadings. Each coordinate in the scores plot represents an individual sample, and each point in the loadings plot represents one spectral region. Therefore, the loadings plot indicates the spectral variables of metabolites that most strongly influence patterns in the scores plot. Similarities and differences between samples can be observed in the scores plots, while spectral regions responsible for the differences can be viewed in the corresponding loading plots. OSC, one of the most frequently used data-filtering methods, is advantageous in analyzing ^{1}H NMR spectroscopic data because it removes variations not correlated to the biological response of interest, such that subsequent PLS models calculated are focused solely on discriminating between elected classes.

Relative changes from identified metabolites contributing to the control and cysteamine-supplemented rats were determined and analyzed statistically using one-way analysis of variance (ANOVA) of SPSS 11.0 software (SPSS Inc., Chicago, IL). Duncan's multiple range test was used to compare differences among the treatment groups. A p value of less than 0.05 was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Analysis of ^{1}H NMR Profiles. Typical ^{1}H NMR spectra obtained for urine and serum were shown in Figure 1. The urine responses were manifested as changes in the concentration of endogenous metabolite levels, including succinate, citrate, and hippurate, as a consequence of cysteamine administration relative to the control group. To detect more subtle treatment-related metabolic differences, pattern recognition techniques were applied. The PCA scores plot of the ^{1}H NMR urine data were shown in Figure 2A. This plot displayed the first two PCs and accounts for 85% of the variation in the samples. The corresponding loading plot (Figure 2B) showed that increased

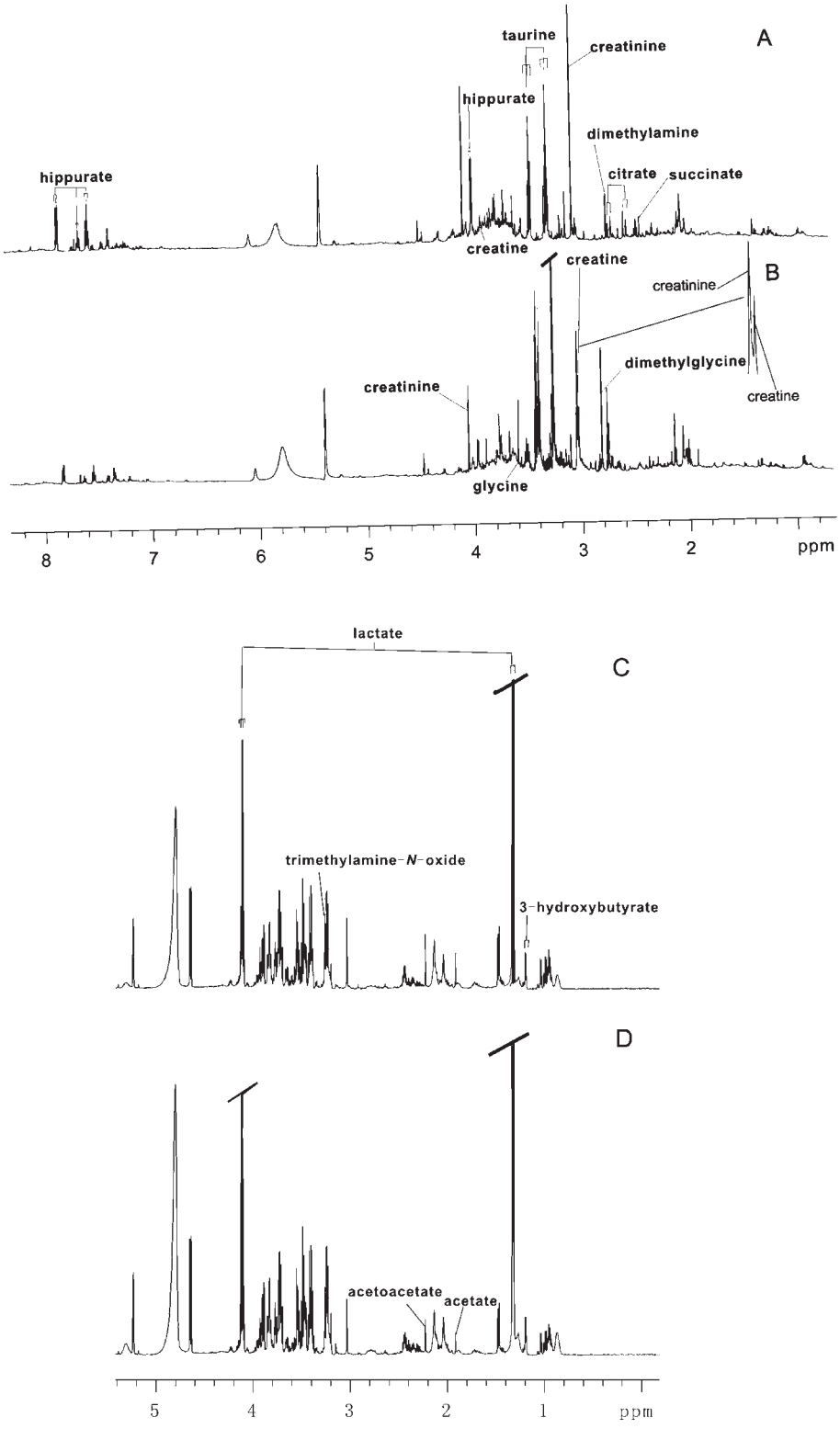

Figure 1. ^{1}H NMR spectra (600 MHz) of urine obtained from rats with the (A) control group and (B) cysteamine-supplemented group and serum obtained from rats with the (C) control group and (D) cysteamine-supplemented group.

urine NMR peaks of dimethylamine, dimethylglycine (DMG), glycine, α-glucose, creatine, and taurine and decreased peaks of creatinine are the major contributors to the separate clustering of the groups. Shown in Figure 2C was PLS results of CPMG data from serum samples with OSC prefiltering. This plot showed the first two PCs and explained 75% of the total variations within the data. Detailed analysis on the loadings plot from Figure 2D indicated that, in the cysteamine group, the content of acetoacetate and trimethylamine-*N*-oxide decreased; however, the content of lactate increased, as compared to the control group.

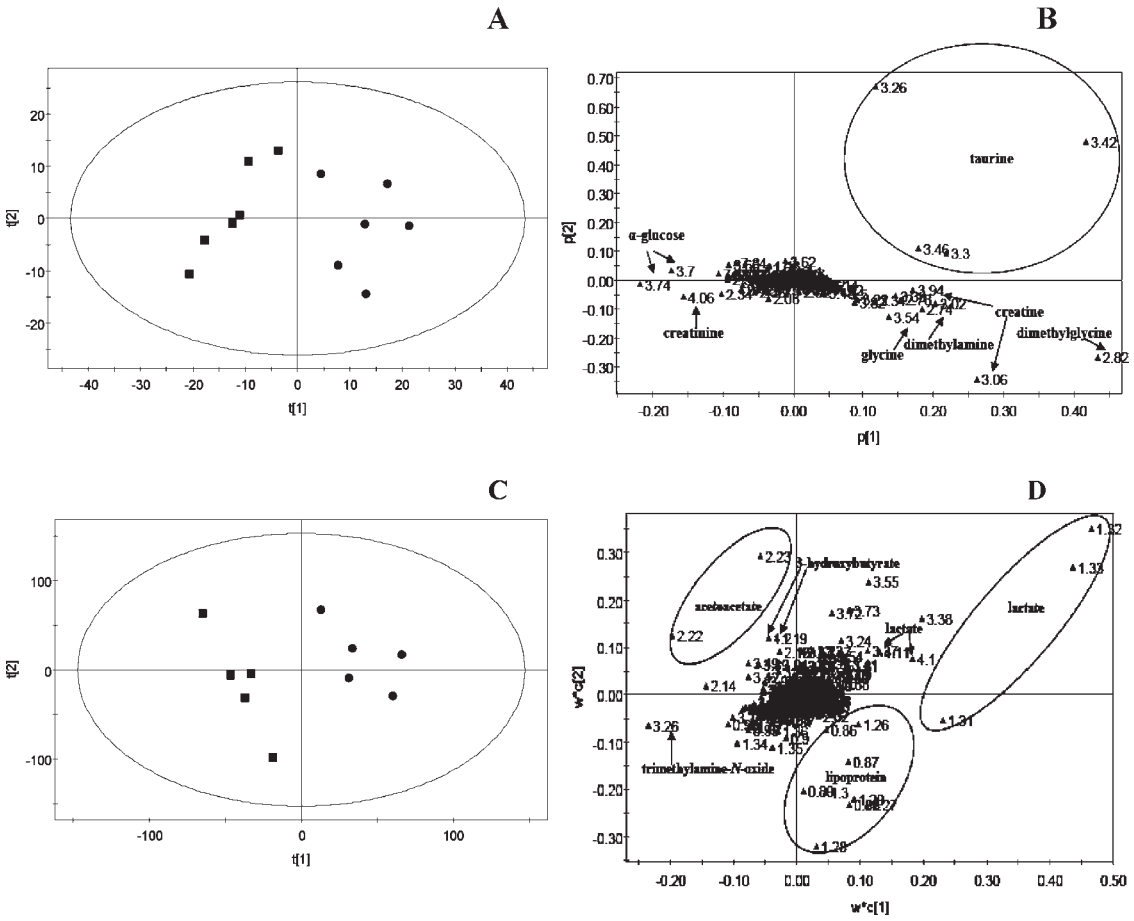

Figure 2. PCA (A) scores plot and (B) loadings plot from ^{1}H NMR spectra of urine and PLS (C) scores plot and (D) loadings plot from ^{1}H NMR spectra of serum obtained from the (■) control and (●) cysteamine-supplemented groups but with OSC prefiltering.

Effects of CS Supplementation. A NMR-based metabolomics analysis was used to demonstrate the metabolic impact of CS supplementation in rats. Examination of PC loadings and subsequent inspection of the corresponding ^{1}H NMR spectra of urine samples enabled identification of endogenous metabolites, whose levels were perturbed by CS exposure. Thus, the approach allowed for the identification of biochemical markers related to CS supplementation. Changes in urine metabolites, as identified by metabolomics and listed in Table 1, imply new concepts on the complex metabolic effects of CS supplementation. A key observation in this study is the impact of CS on intermediary metabolism in rats. Of the identified differential metabolites in the CS treatment group, succinate, an important intermediate in the tricarboxylic acid (TCA) cycle, was decreased. This suggests that the TCA cycle is downregulated by CS supplementation, which is also implied by decreased levels of citrate, another intermediate in the TCA cycle. This could be attributed to several possible explanations. First, decreased urinary levels of TCA cycle intermediates may indicate inhibition of whole-body oxidative energy metabolism. Second, the urinary excretion of TCA cycle intermediates may signal altered regulation of anaplerosis and cataplerosis pathways, that is, the net synthesis and net removal of TCA cycle intermediates from mitochondria, respectively. These pathways play a role in biosynthesis routes, such as fatty acid biosynthesis in the liver, gluconeogenesis in the liver and kidney cortex, and glyceroneogenesis in adipose tissue,[16] as well as in insulin secretion stimulation.[17] Third, decreased TCA cycle activity may indicate changes in liver and kidney functions,[18] because the chemical shifts identified were indicative of components involved in cellular metabolism.

Table 1. Observed Changes in Urine Metabolites Obtained from Chronic Cysteamine Supplementation Rats Compared to Control Rats

NMR chemical shift (δ)	metabolites	change in direction[a]	p value[b]
2.42	succinate	↓	<0.01
2.74	dimethylamine	↑	<0.01
2.54, 2.70	citrate	↓	<0.01
2.82	dimethylglycine	↑	<0.01
3.54	glycine	↑	<0.01
3.26−3.3, 3.42−3.46	taurine	↑	<0.05
3.70, 3.74	α-glucose	↑	<0.01
3.94, 3.06	creatine	↑	<0.01
4.06, 3.06	creatinine	↓	<0.01
7.82, 7.66, 7.54, 3.98	hippurate	↓	<0.05

[a] ↓ indicates a relative decrease, and ↑ indicates a relative increase in the integral value for the region containing the identified metabolite. [b] p values for the changing metabolites were assessed using ANOVA in SPSS 11.0 software based on the integrals of the selected peaks. p values are significant at the <0.05 or <0.01 level.

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Table 2. Changes in Relative Concentrations of Serum Metabolites in Cysteamine-Supplemented Rats Compared to Control Rats

NMR chemical shift (δ)	metabolites	change in direction[a]	p value[b]
1.19−1.20	3-hydroxybutyrate	−	0.118
1.31−1.33, 4.10−4.11	lactate	↑	<0.05
1.92	acetate	↓	<0.01
2.22−2.23	acetoacetate	↓	<0.01
3.26	trimethylamine-*N*-oxide	↓	<0.05

[a] ↓ indicates a relative decrease; ↑ indicates a relative increase; and − indicates no change in the integral value for the region containing the identified metabolite. [b] p values for the changing metabolites were assessed using ANOVA in SPSS 11.0 software based on the integrals of the selected peaks. p values are significant at the <0.05 or <0.01 level, and p values are not significant at >0.05.

In obvious contrast to a human study, a recent study in rats shows a decreased urinary excretion of some TCA cycle intermediates (citrate and 2-oxoglutarate) after epicatechin administration.[19] This animal study was possibly flawed by the very high epicatechin dose and/or animal conditioning[19] because reduced urinary citrate levels have been suggested as indicators of kidney dysfunction.[20] An increased lactate concentration was also observed in the serum of CS-supplemented rats. This is in agreement with our previous study that CS supplementation (70 mg/kg) increased serum lactate dehydrogenase of finishing pigs (data not shown). Lactate is associated with energy metabolism and is the end product of compounds involved in energy metabolism. An increased lactate level is related to increased anaerobic glycolysis. In addition, the increase in the serum lactate level implies that gluconeogenesis is inhibited and that carbohydrate and energy metabolism was altered. Acetoacetate and 3-hydroxybutyrate are products of fatty acid oxidation in the liver, and their ratios are useful indicators of the mitochondrial redox state.[21] Cysteamine supplementation decreased concentrations of acetoacetate but did not change concentrations of 3-hydroxybutyrate in serum (Table 2), thus reducing the acetoacetate/3-hydroxybutyrate ratio. This suggests a less oxidized state of cells, which can be brought about by less oxidation of fatty acids. When the results are taken together, they indicate reduced energy expenditure in CS-supplemented animals.

Another novel and unexpected finding from this work is that serum concentrations of short-chain fatty acids (acetate) and nitrogenous products (trimethylamine-*N*-oxide) were decreased in CS-supplemented rats (Table 2). Urinary excretion of dimethylamine was also decreased. Notably, they are microbial metabolites of carbohydrates and amino acids,[22,23] which are possibly produced in the lumen of small and large intestines. Intestinal microbes convert dietary non-digestible fibers into short-chain fatty acids and other nutrients that can be used by the mammalian host as energy sources and precursors for fatty acid synthesis.[24] The introduction of CS into the mammalian system may displace baseline mammalian-to-microbial behavior, thereby causing a disruption in microbial populations and, hence, metabolism. Changes in these metabolites may result from a reduced number and/or altered activity of intestinal microorganisms.[25] An *in vitro* experiment has revealed that CS can inhibit some microbes[26] to reduce their number and/or metabolic activity. The reduction in the number of intestinal bacteria is believed to be associated with decreased fat accretion,[27] although the underlying mechanisms are not known. Results of the current study also indicated that CS induced a decrease in urinary excretion of hippurate, which results from both renal and hepatic synthesis of glycine and benzoic acid and is also the degradation product of flavonols acted upon by intestinal microoganisms.[28] Variations in the urinary hippurate concentration have also been linked to changes in the distribution of intestinal microbial colonies.[29] Mammalian metabolism is known to be significantly affected by its interaction with the complex gut microbial community.[30] Gut microbiota exert a profound impact on the development and structure of the intestinal epithelium, the digestive and absorptive capabilities of the intestine, and the host immune system.[31] Possible disturbances of gut microbiota by CS administration may be expected to have an impact on health status. Microbiological identification of specific changes in the microbiota community may be helpful in addressing the metabolic implications of CS supplementation. Cysteamine supplementation was also found to induce an increase in urinary excretion of taurine, which functions as renal osmolytes. The main route of CS metabolism is thought to involve oxidation to hypotaurine followed by oxidation to taurine via sequential reactions catalyzed by specific monooxygenases.[32] Thus, CS supplementation increases the taurine concentration, which is consistent with previous results.[33] Finally, urinary excretion of DMG was increased by CS supplementation. Dimethylglycine is produced in cells as an intermediate in the metabolism of choline to glycine. It is said to function as a key intermediary in the biological pathway that supplies the body with methyl groups.[34] Dimethylglycine also acts as a detoxifying agent and antioxidant, protecting body cells from unwanted reactions caused by free radicals.[35,36] Other claims include DMG as an energy booster and a stess reducer.[37,38] Thus, CS supplementation may help in health benefits. Dimethylglycine is metabolized in the liver to monomethylglycine or sarcosine, which, in turn, is converted to glycine.[34,38] This is in agreement with the current study that urinary excretion of glycine was increased.

In conclusion, a NMR-based metabolomics analysis indicates that chronic CS administration can induce profile changes in the urine and serum, as well as a perturbation of energy metabolism. Results also suggest that chronic CS can modulate intestinal microbial metabolism in rats. To our knowledge, this is the first *in vivo* report on the response of animal biological systems to long-term CS supplementation. Future studies may be directed toward a mechanistic understanding of the long-term effects of chronic CS on animal intermediary metabolism.

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ABBREVIATIONS USED

CS, cysteamine; FID, free induction decay; ^{1}H NMR, proton nuclear magnetic resonance; PCA, principal component analysis; PLS, projection to latent structures; OSC, orthogonal signal correction; TCA, tricarboxylic acid; DMG, dimethylglycine; TSP, 3-(trimethylsilyl)propionic-(2,2,3,3-d_4) acid sodium salt

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