NMR METABOLIC PROFILING COMBINED WITH TWO-STEP PRINCIPAL COMPONENT ANALYSIS FOR TOXIN-INDUCED DIABETES MODEL RAT USING URINE

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ABSTRACT — Nuclear magnetic resonance-based metabolic profiling (NMR-MP) was applied to evaluate disorder model animals using urine. Diabetic nephropathy was established in this experiment by administering streptozotocin to Wistar rats, which immediately developed diabetes after toxin-treatment and then gradually produced albumin-containing urine (albuminuria). Urine samples were collected for the first 4 weeks after toxin treatment. Predominant urinary sugar signals were seen in 1H-NMR spectra of diabetes rat urine, and spectra were processed and subjected to multivariate analysis. Principal component analysis (PCA) identified 3 outliers among 20 individuals. Outlier rats did not develop urinary sugar and were later found to be rats insufficient to establish diabetes models. A second PCA was performed excluding the additional glucose-signal region (3.2-6.3 ppm), as glucose signals had a predominant effect that may mask details of other metabolic profiles. Consequently another outlier was revealed. This exceptional rat did not develop albuminuria even after producing glucosuria for 14 weeks. NMR metabolic profiling provides good guidance to evaluate biophysical conditions of animals, enabling detection of abnormalities in the early stage of toxicological experiments.

KEY WORDS: NMR, Metabolic profiling, PCA, Diabetes, Streptozotocin, Rat

INTRODUCTION

The utility of NMR-based metabolic profiling (NMR-MP) methodology has been demonstrated in toxicological fields, such as in the research of nephrotoxins (Portilla et al., 2006; Lenz et al., 2005; Anthony et al., 1994; Holmes et al., 1992). This technique provides a method of monitoring xenobiotics and clinical toxicology (Zhang et al., 2006; Wu et al., 2004), and has been coupled with several pattern recognition analyses. Among these, PCA is widely used to classify NMR-derived data. PCA provides good representation for time-related responses in metabolic composition variance as a method of monitoring the progression of toxicity and recovery (Lindon et al., 2003).

Diabetes is a serious and widespread human disease and diabetic nephropathy is a well-known complication resulting from kidney breakdown. Animal models are indispensable for studying disease progress, including various biological responses. The present
experiment was performed to establish diabetic nephropathy model rats by introducing streptozotocin. Various biochemical and physical characteristics of each rat, such as urine volume, blood sugar levels, urinary albumin levels and body weight were measured every week to check toxic effect, but were yet insufficient to identify disease progress in the very early stages. We performed NMR-MP using rat urine in the initial stage of the experimental period and succeeded in detecting and assessing physiological changes in each rat that reacted based on complex individual multi-factorial metabolic backgrounds. The present study identified urine NMR-MP coupled with 2-step PCA as a useful tool to detect abnormalities in toxin-induced animals in the very early stages.

**MATERIALS AND METHODS**

**Experimental**

Diabetic nephropathy was induced in male Wistar rats (11-weeks-old, 230-250 g, n=15; Japan SLC, Hamamatsu, Japan), as follows. Left unilateral nephrectomy through a flank incision was performed on each rat under sodium pentobarbital anesthesia (45 mg/kg body weight). At 5-7 days after nephrectomy, rats received an intravenous injection of streptozotocin (15 mg/kg body weight; Sigma Chemical, St. Louis, US) via the tail vein. The drug was freshly dissolved in 50 mM Na-citrate buffer (pH 4.5, 15 mg/ml) just before injection. Control animals received unilateral nephrectomy and an injection of vehicle alone (n=5). All 20 animals were maintained on a standard rat diet and were provided with ad libitum access to water throughout the 14 weeks of the experiment.

Blood sugar levels in toxin-treated rats started high immediately and remained high throughout the whole experiment. Urinary albumin was measured by enzyme-linked immunosorbent assay (ELISA) as described previously (Weise et al., 1993). This procedure was performed in accordance with the guidelines

![Fig. 1](image-url) **Fig. 1.** The examples of ‘H-NMR spectra from urine. The upper 3 spectra are toxin-induced: a) Rat 1 in week 1; b) Rat 15 in week 1; c) Rat 3 in week 2, and d) control. Note that Rat1 was found to be a unique and particular animal after the second PCA was applied. Water (*) and urea(***) regions(4.3-6.3 ppm) were removed from datasets. The arrows indicate the overflowed glucose signals.
Urinary albumin levels started to increase around 6 weeks after toxin injection, then gradually increased with development of nephropathy. To obtain 24-hr urine samples, animals were housed individually in metabolic cages once a week without feeding. Fresh urine was immediately stored in a freezer (−30 °C) until workable amounts had been accumulated. Urine samples used in NMR measurement were collected every week during the initial 4 weeks that animals produced glucosuria without albumin.

NMR spectroscopy and statistical analysis

Thawed urine samples preparation and acquisition of 1H-NMR spectra have been described elsewhere.

Fig. 2. First PCA. Upper score plot demonstrates 2 groups along the PC1 axis, where circle dots and open triangles represent control and toxin-induced disease, respectively. a-d) Corresponding to spectra in Fig. 1, respectively. The circled region of the broken line in the loading plot (lower) represents marker variables, which correspond to glucose signals for classification of the two groups.
(Fujiwara et al., 2005). All NMR raw data (fids) were processed by ALICE2 for Metabolome software (version 1.0; JEOL, Tokyo, Japan). Each spectrum was automatically segmented in 0.04 ppm regions, and each region was integrated and then normalized over the total sum of integrals to give a numeric dataset. The range of 4.3-6.3 ppm containing residual water and urea signals was excluded from the dataset. With these minimum settings, the software automatically performed PCA. Datasets were represented as data points in 2 dimensional (2D)-score and 2D-loading plot, respectively. Briefly, score plot represents the distribution and separation of each dataset, loadings stand for its contribution to separation. The second PCA was performed after excluding the glucose-signal region including residual water and urea (3.2-6.3 ppm) from the datasets, i.e., datasets contained 2 regions of 0.5-3.2 ppm and 6.3-9.5 ppm.

RESULTS AND DISCUSSION

A total of 80 urine samples from 20 rats over 4 weeks were used in this study. In the 1H-NMR spectra shown in Fig. 1, the overflowing peaks were glucose signals that predominated from 1 week after toxin injection (12-weeks-old). The PCA score plot and loading plot are presented in Fig. 2. PC1 and PC2 explained 87.8% and 6.6%, respectively. The distribution/separation of data points were explained mostly by PC1 value. The two clusters by data points appeared along the PC1 axis, with all points from controls in the right-side cluster and almost all toxin-treated samples in the left-side aggregated cluster. Arrows on the score plot in Fig. 2 corresponded to each spectrum in Fig. 1, demonstrating that the clear discrimination between right and left clusters were explained by the presence of urinary glucose. From the loading plot in Fig. 2, variables responsible for the classification corresponded to glucose signals (3.2-6.3 ppm).

The score plot in Fig. 3 was delivered from datasets from 60 urine samples (15 streptozotocin-treated rats), excluding controls. Most data points of the 15 rats stayed on the left side of the plot, but 4 represented outliers. By checking 4-week trajectories

![Fig. 3](image)

Fig. 3. First PCA. Trajectories of 4 rats (Rats 5, 6, 9 and 11) over 4 weeks. For further detail, see text.
(time courses) of these outliers (arrow marks, Fig. 3), 2 rats (Rats 5 and 9) moved to the left once and then reached to the right side again on week 4, representing recovery from toxin-induced glucosuria. Rat 6 remained on the right side, and did not develop glucosuria at all.

One rat (Rat 11) was initially in week 1 located at the right side and then entered the left cluster of glucosuria, which can be interpreted as a response delay. Consequently, the 3 rats (Rats 5, 6 and 9) were concluded to represent outliers from first-step PCA. These rats were finally considered insufficient as diabetes models and removed from experimentation, killed after several weeks rearing. First-step PCA in NMR-MP can thus detect and assess the process of establishment for each diabetic model rat.

Second-step PCA was performed to obtain further information. Analysis was performed after remov-

![Fig. 4.](image)

Second PCA. In the upper score plot, stars combined with arrows show time course for Rat 1. In the loading plot, arrowed variables represent markers for classifying Rat 1 and others. Numeric numbers are chemical shifts of corresponding buckets (bins) in NMR spectrum.
ing the predominating glucose-signals from datasets. Glucose signals had a major effect (PC1 > 85%) that was likely to mask other metabolic profiles (Figs. 1, 2). For the sake of drawing out hidden details, glucose signals (3.2-6.3 ppm) were ignored. The resulting PCA score plot with values of 46.6% (PC1) and 29.5% (PC2), resulted in a single extraordinal outlier, Rat 1 (Fig. 4). This trajectory indicates exceptional behavior among other rats in the early stage even from 2 weeks after injection. The loading plot in Figure 4(lower panel) represents the marker candidate variables to classify this particular rat from other rats. By checking corresponding NMR spectrum, we observed increase of signals which indicated a hydroxyl-related CH₃, CH and CH₂. The ratio of these signals in each spectra was identical, standing for the partial structure of a biomarker. Of note is the fact that the loading plot coincides with the abnormal plot concluded from other biochemical data collected throughout the experiment.

When looking into the biochemical and physiological data from the entire experiment, beside the outlier rats in first-step PCA (Rats 5, 6 and 9), the remainder were all accepted as suitable disease models that developed glucosuria according to high blood sugar. These rats subsequently developed high albuminuria gradually after around 6 weeks and developed nephropathy. By checking body weight, all values gradually decreased slightly. Conversely, all 5 controls gained about +40% weight during the experiment, as rats were in the growth period.

However, at second-step PCA, we found another abnormal rat that was finally found to be exceptional, not developing albuminuria even after 14 weeks of high blood sugar and glucosuria. In addition, weight loss in Rat 1 was less than that in other diabetic nephropathy rats, despite the absence of nephropathy (data not shown).

To identify biomarker candidates that may correlate to essential characteristics of glucose resistance due to the reaction(s) of single or multiple organs, and to understand the physiological reasons underlying Rat 1, we are currently implementing further detailed analyses.

In general, subacute toxin often invokes substantial adverse effects on metabolic profile that mask other details of metabolites. It is significant to analyze with selected proper and efficient variables in the second statistical analysis process. From this point, in this analysis, Rat 1 remained during 4 weeks inside 1 cluster in the first-step PCA in Fig. 2, but was not discriminated by even higher components of PCs than PC1 and PC2. Importantly, variable selection by excluding glucose signals enabled differentiation of this rat from other rats. This novel and useful technique, 2-step PCA in NMR-MP, can provide comprehensive information on complicated situations.

NMR-MP properly assessed the process of making model animals, enabling detection of two kinds of insufficient model animals in the early stages, reducing rearing cost. In pattern recognition analysis, variable selection provides a powerful tool to extract useful information from small sample sizes, which are often encountered in biomedical studies (Nikulin et al., 1998). In our final PCA, available variables were selected by deletion of dominant-signal variables. Variable selection can also be applied using the SIMCA method (Fujiwara et al., 2006), and which method should be applied is case-dependent. In conclusion, NMR-MP using 2-step PCA offers a sensitive method for detecting abnormalities in toxin-induced metabolic changes, and has promising potential to identify symptoms of physiological changes or diseases in the very early stages.

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Diabetes rat urine NMR metabolic profiling.