INTEGRATED NMR-BASED METABONOMIC INVESTIGATION OF EARLY METABOLIC EFFECTS OF ETHYLENE GLYCOL MONOMETHYL ETHER (EGME) ON MALE REPRODUCTIVE ORGANS IN RATS

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ABSTRACT — High-resolution Magic Angle Spinning (Hr-MAS) ¹H-NMR spectroscopy was used to analyze intact testicular tissues *ex vivo* and to investigate the toxicological effects of ethylene glycol monomethyl ether (EGME), a well-known spermatocytes toxicant, on male reproductive organs by NMR-based metabonomic analysis. Especially, we reported the first Hr-MAS ¹H-NMR spectra of epididymis. Sexually matured male rats were treated with 50 and 2,000 mg/kg EGME, and body weight, reproductive organs weight, histopathology and plasma biochemistry were examined at 6 and 24 hr after administration. Two multivariate statistical methods, namely, unsupervised PCA and supervised PLS-DA, indicated that the balance of endogenous metabolites was perturbed in both reproductive organs and biofluids. In the testes, lactate, creatine and glutathione were mainly affected by EGME treatment. In urine and plasma, altered excretions of the TCA cycle intermediates (2-oxoglutarate, citrate and succinate) and the ketone-bodies (acetoacetate and β -hydroxybutyrate) were also observed. The finding in current integrated metabonomic analysis of both intact tissues and biofluids suggested that EGME-induced testicular toxicity was attributed to perturbation of the energy supply processes, suppression of the TCA cycle, or oxidative stress. Furthermore, Hr-MAS ¹H-NMR proved useful to investigate the molecular snapshot of biological tissues and the mechanism of toxicity.

KEY WORDS: Metabonomics, Hr-MAS NMR, Ethylene glycol monomethyl ether, Rat, Testicular toxicity

INTRODUCTION

Ethylene glycol monomethyl ether (EGME) is widely used as a solvent for varnishes, dyes, paints and printing inks. In the industry, EGME is extensively utilized in the manufacture of photographic film and as an anti-freeze in jet-fuels. EGME is readily absorbed, either percutaneously or by inhalation, into the human body due to its volatile nature. Hence, undesirable adverse effects on several organs have been reported. Male reproductive toxicity due to EGME, one of the critical adverse events, was described more than 60 years ago (Wiley *et al.*, 1936). Studies in laboratory animals showed that EGME induced testicular atrophy

and impaired infertility (Chapin *et al.*, 1984; Anderson *et al.*, 1987; Foster *et al.*, 1983, 1984). In the testes, the primary and pachytene spermatocytes were known as targets of EGME, while early and late stage spermatids, and Sertoli cells did not appear affected (Chapin and Lamb, 1984).

Several “omics” investigations have been conducted to elucidate the molecular basis of testicular damage induced by EGME. Transcriptomic analysis revealed that several genes, which had important roles in spermatogenesis, were differentially expressed in testes after EGME treatment (Wang and Chapin, 2000; Fukushima *et al.*, 2005). Syed and Hecht (1998) also demonstrated that EGME induced changes in gene

expression in Sertoli cells as well as in spermatocytes. Interestingly, they detected up-regulation of the gene coding oxidative stress-responsive protein in Sertoli cells, despite these cells not being considered a target of EGME. Although there were few reports regarding global protein expression (proteomics), spermatogenesis-related and oxidative stress-inducible proteins were found to be affected by several reproductive toxicants, including EGME (Yamamoto *et al.*, 2005). In this context, studies on the content of endogenous metabolites and their alterations will further clarify biological processes.

Nuclear magnetic resonance (NMR)-based metabonomics is a relatively new technique used to comprehensively investigate biological processes, as a next generation of 'omics' technology (Lindon *et al.*, 2000). A considerable benefit of metabonomic analysis is that the findings directly link to the responses seen *in vivo*. Metabonomics is usually conducted on biofluids, mainly urine, plasma and serum. Therefore, one of the key advantages of metabonomics is that samples can be obtained non-invasively and the time course of alterations can be monitored within individual animals. In contrast, it is often difficult to identify the affected organ because analytes are either urine or plasma. High-resolution Magic Angle Spinning (Hr-MAS) is one of the "solid-state" ^1H -NMR techniques, which enables direct monitoring of the metabolic state of various intact biological tissues. Hr-MAS ^1H -NMR spectroscopy has recently been successfully used on several intact tissues in the following studies: liver (Waters *et al.*, 2001; Coen *et al.*, 2003; Leliott *et al.*, 2005); kidney (Garrod *et al.*, 1999, 2001); gut (Martin *et al.*, 2007); lung (Azmi *et al.*, 2005); lens (Tessem *et al.*, 2006); gastric mucosa (Schenetti *et al.*, 2006) and brain (Cheng *et al.*, 1997). Integrated metabonomic analyses by both Hr-MAS ^1H -NMR of tissues and "liquid-state" ^1H -NMR of biofluids will provide novel knowledge related to biological and toxicological responses in the body.

The present study was aimed to monitor the details of metabolic perturbation, when testes were damaged by a single dose of EGME. Using NMR-based metabonomic analyses, we investigated the effects of EGME on endogenous metabolites in reproductive organs and biofluids. In summary, we successfully detected several changes in endogenous metabolites in testes, epididymides, urine and plasma. The findings suggested that EGME-induced testicular toxicity resulted from either alterations of the energy metabolism, TCA cycle, or oxidative stress. Besides,

integrated evaluation of endogenous metabolites in tissues and biofluids revealed by "solid-state" and "liquid-state" NMR-based metabonomics proved useful to evaluate mechanisms of toxicity.

MATERIALS AND METHODS

Animals and treatment

The present study was conducted under the approval of the Animal Ethics Committee (Institute of Animal Care & Use Committee of Pfizer Inc.). Twelve-week-old Sprague-Dawley male rats [Crj:CD(SD)IGS] were purchased from Charles-River Laboratories Japan, Inc. (Yokohama, Kanagawa, Japan). The animals were acclimated to our laboratory for 1 week, and then randomly assigned to each treatment group by body weight. The animals were housed individually in metabolic cages and maintained under controlled room temperature (21–25°C) and lighting (12-hr light and dark cycle). The animals were given access to municipal drinking water and an animal diet *ad libitum*. In the present study, ethylene glycol monomethyl ether (EGME) (Sigma, St. Louis, MO, USA) was used as the male reproductive toxicant. Individual animals were administered EGME by oral gavage at doses of 50 (low-dose) and 2,000 mg/kg (high-dose) using a Teflon sonde. The dosing solution of EGME was prepared by dilution with distilled water. The animals assigned to the control group were given distilled water. Five animals each were assigned to the control and treatments groups. Six and 24 hr after the administration, the control and EGME-treated animals were euthanized under CO_2 inhalation, and then blood and the reproductive organs were removed. The body and reproductive organs weight was recorded.

Sample collection

As for the 24 hr treatment group, urine was collected into conical tubes containing 1% (w/v) sodium azide (Wako Pure Chemicals, Osaka, Japan) from animals housed in metabolic cages. The tubes were maintained under cold conditions during the collection of urine. The periods for sampling of urine were as follows: –24 to –16 hr, –16 to 0 hr (pre-dose), 0 to 8 hr and 8 to 24 hr (post-dose). Blood and reproductive organs were collected at necropsy 6 and 24 hr after the administration. Plasma was immediately separated by centrifugation at 3,000 rpm for 10 min at 4°C. The left testis of each animal was fixed in 4% paraformaldehyde for histopathological examination. The right testis and epididymis were immediately frozen in liquid

nitrogen. These samples were stored below -80°C for subsequent clinicopathological and metabonomic analysis.

Plasma biochemistry

The following parameters were measured: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (GGT), total bilirubin (TB), blood urea nitrogen (BUN), creatinine (CREA), total cholesterol (T-CHOL), and total protein (TPR). The data were obtained using the clinical automatic analyzer H-7070 (Hitachi, Ltd., Tokyo, Japan).

Testes histopathology

One testis from each animal was examined. The fixed testes were embedded in paraffin and blocks were prepared. Testicular sections were obtained from each testis at 5 μm thickness, then stained with hematoxylin and eosin (Wako Pure Chemicals), and examined by light microscopy.

Hr-MAS ^1H -NMR spectroscopy of reproductive organs

Reproductive organs, testes and epididymides, collected at the necropsy at 24 hr after EGME administration were subjected to “solid-state” Hr-MAS ^1H -NMR spectroscopic analysis. Samples of reproductive organs (10 to 20 mg) were placed in 4 mm outer diameter zirconia rotors (Bruker Biospin GmbH, Rheinstetten, Germany), and then 50 μL of D_2O (ISOTEC Inc., Miamisburg, OH, USA) containing 1 mM sodium 3-trimethylsilyl-(2,2,3,3- $^2\text{H}_4$)-1-propionate (TSP- d_4 , ISOTEC Inc.) was added. TSP and D_2O provided the chemical shift references (δ_{H} 0.0) and a field-frequency lock signal for the NMR spectrometer in acquisition of free induction decay (FID), respectively. Hr-MAS ^1H -NMR measurements were made on a Bruker DRX600 NMR spectrometer (Bruker Biospin GmbH) operating at 600.13 MHz ^1H frequency at a MAS spin rate of 5,000 Hz. Hr-MAS spectra were acquired at ambient probe temperature (300 K). The one-dimensional T_2 -edited Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence [RD (relaxation delay)- $\pi/2_x$ -(τ - π_y - τ) $_n$ -acquisition] with standard presaturation of the water resonance was applied to the measurements. Here, τ and n represent the refocusing delay time and number of cycles, respectively. These parameters were set to 1 ms and 30 cycles, respectively; thus, the total spin-echo time ($2n\tau$) was calculated to be 60 ms.

^1H -NMR spectroscopy of biofluids

Urine and plasma were subjected to “liquid-state” ^1H -NMR spectroscopy. Aliquots of urine were added to half volume of the buffer (0.2 M sodium phosphate buffer ($\text{H}_2\text{O}/\text{D}_2\text{O}$; 90/10) containing 1 mM TSP- d_4 and 3 mM sodium azide) to stabilize the urine pH. On the other hand, aliquots of plasma were mixed with two volumes of 0.9% (w/v) NaCl (Wako Pure Chemicals) in $\text{H}_2\text{O}/\text{D}_2\text{O}$ (90/10) solution. After vigorously mixing both samples, they were allowed to stand for a few minutes and then centrifuged at 10,000 rpm for 5 min to remove insoluble materials and visible particles. One-dimensional ^1H -NMR spectra of urine and plasma were acquired using the Bruker DRX600 NMR spectrometer (Bruker Biospin GmbH) at 600.13 MHz ^1H observation frequency at ambient probe temperature (300 K). Standard presaturation pulse sequence for water signal suppression was used; namely, nuclear Overhauser effect correlated spectroscopy (NOESY)-presaturation for urine samples and CPMG-presaturation for plasma samples. Chemical shifts were referenced to TSP at 0 ppm and the glucose doublet signal at 5.23 ppm for urine and plasma spectra, respectively.

NMR spectral data processing

Using XWIN-NMR software (Ver. 2.5, Bruker Biospin GmbH), Fourier transformation was made for all FIDs acquired, and then ^1H -NMR spectra were manually corrected for phase and baseline distortions. The appropriate spectral region (reproductive organs: δ 0.2-9.0; urine: δ 0.2-10.0; plasma: δ 0.2-6.0) was integrated into buckets 0.04 ppm wide (“bins”) using AMIX program (ver. 3.6.8, Bruker Biospin GmbH) to reduce data complexity of the spectra. The region corresponding to water resonance in the spectrum (reproductive organs: δ 4.70-5.10, urine: δ 4.50-5.40, plasma: δ 4.66-5.06) was removed from the analysis. In addition, the region of urea resonance (δ 5.40-6.00) was also set to zero integral value for only urine spectra. To avoid including xenobiotic-related metabolites in the analysis, the dosed compound related resonances were also omitted for urine and plasma spectra. The integration values of the remaining spectral region were divided by the total intensity to normalize the value (global normalization) before pattern recognition analysis.

Multivariate statistical analysis in metabonomic analysis

The data sets of “bins” NMR spectra were imported into the SIMCA-P software package (ver.

11.5, Umetrics AB, Umea, Sweden) for multivariate statistical analyses as pattern recognition. To evaluate the changes in patterns derived from the differences in the content of endogenous metabolites, unsupervised *principal component analysis* (PCA) and supervised *projections to latent structures by means of partial least squares-discriminant analysis* (PLS-DA) were applied to the analysis of all spectra. Prior to data analysis, the variables were mean-centered and Pareto scaled, that is, each variable was weighed by the square root of its standard deviation. At first, PCA using the entire data set was performed to indicate trends of group characteristics and time course, outliers, and any other systemic pattern within the analysis. Next, PLS-DA was conducted to focus on the classification and discrimination between the treatments classes (e.g. control versus treatment). PLS-DA was directed at determining bins that indicated meaningful endogenous metabolites, in the data that correlated with these known classes.

Statistical analysis

Body weight, reproductive organs weight and parameters of plasma biochemistry were expressed as the mean $\pm$ SD of 5 rats, and statistical significance was determined by Dunnett's multiple comparison procedure (Gad and Weil, 1994). A probability of less than 5% was considered to be statistically significant.

RESULTS

Body and reproductive organs weight

Table 1 shows the body weight and reproductive organs weight at 6 and 24 hr after treatment. There were no remarkable changes in body and reproductive organs weight at 6 hr after the administration of EGME. At 24 hr, significant decreases in body weight were observed only in the high-dose group. Regarding reproductive organs, the weight of testes from rats

administered the high-dose EGME tended to decrease, but the difference with the control group was not statistically significant.

Plasma biochemistry

Except for ALT, ALP and BUN in the high-dose group, most clinicopathological parameters did not change remarkably (Table 2). An elevation of BUN was observed in the high-dose at 24 hr after administration, whereas no change in plasma creatinine levels was detected. In addition, no change in urinary protein levels was observed at this time point compared with the levels in the control group (data not shown). Therefore, the slight elevation of BUN might not have been due to the kidney dysfunction.

Testes histopathology

Testes were histopathologically examined at 6 and 24 hr after the administration of EGME (Fig. 1). Histopathological changes were detected only in the high-dose group. Slight degeneration was observed in some pachytene spermatocytes at 6 hr (data not shown). At 24 hr, degenerative changes were observed in spermatocytes. On the other hand, there were no remarkable histopathological changes related to the administration of EGME at low dose.

Hr-MAS ¹H-NMR spectra of reproductive organs

Hr-MAS ¹H-NMR spectroscopic analysis provided the snapshots of metabolic profiles in reproductive organs excised from the same individual rat. Fig. 2 shows the typical spectra of a testis, and caput and cauda regions of the epididymis. The Hr-MAS ¹H-NMR spectrum of testis was characterized by high contents of creatine and phosphatidylcholine (Fig. 2A). On the other hand, the epididymis showed different profiles of endogenous metabolites in comparison with testis. The spectra (Figs. 2B and 2C) showed that endogenous metabolites in the epididymis were altered

Table 1. Body weight and reproductive organs weight of male rats at 6 and 24 hr after the administration of EGME.

	6 hr						24 hr					
	Control		EGME				Control		EGME			
			50 mg/kg		2,000 mg/kg				50 mg/kg		2,000 mg/kg	
Body weight (g)	392.6	± 18.3	396.8	± 17.1	395.6	± 24.2	410.5	± 21.2	397.0	± 25.3	373.3	± 11.4*
Testis (g)	3.167	± 0.119	2.957	± 0.278	3.018	± 0.203	3.065	± 0.291	3.013	± 0.282	2.912	± 0.077
Epididymis (g)	1.148	± 0.070	1.157	± 0.071	1.152	± 0.056	1.053	± 0.061	1.145	± 0.130	1.140	± 0.092

Each value represents the mean $\pm$ S.D. of 5 rats.

[*] indicates statistically significant difference from the control group ($p < 0.05$, Dunnett's multiple comparison).

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Table 2. Clinical pathology of plasma from male rats administered a single dose of EGME at 6 and 24 hr after the administration.

	6 hr						24 hr					
	Control	EGME				Control	EGME					
		50 mg/kg		2,000 mg/kg			50 mg/kg		2,000 mg/kg			
GGT (U/L)	0.6 ± 0.5	0.6 ± 0.5	0.4 ± 0.5	0.5	1.0 ± 0.0	1.0 ± 0.0	0.6 ± 0.5					
AST (U/L)	76.6 ± 10.3	75.2 ± 5.0	81.4 ± 9.4	9.4	84.6 ± 14.0	92.0 ± 25.9	126.2 ± 54.2					
ALT (U/L)	35.6 ± 3.6	39.4 ± 1.8	43.4 ± 4.9*		43.6 ± 9.0	35.2 ± 9.7	47.0 ± 21.2					
ALP (U/L)	420.2 ± 88.2	468.2 ± 128.2	468.6 ± 100.0		492.2 ± 80.4	379.4 ± 26.1*	295.8 ± 32.2*					
TB (mg/dL)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0					
BUN (mg/dL)	12.8 ± 2.5	12.8 ± 2.2	14.8 ± 0.8		13.8 ± 1.8	14.0 ± 1.6	22.4 ± 3.7*					
CREA (mg/dL)	0.3 ± 0.1	0.2 ± 0.0	0.0 ± 0.1	0.1	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1					
CHOL (mg/dL)	58.0 ± 10.9	65.8 ± 8.9	74.2 ± 13.7		69.2 ± 12.6	65.6 ± 9.2	50.2 ± 10.0					
TPR (g/dL)	5.8 ± 0.3	6.1 ± 0.3	6.7 ± 0.2	0.2	6.0 ± 0.4	6.1 ± 0.4	6.8 ± 0.3					

Each value represents the mean ± standard deviation (SD) of 5 rats.

[*] indicates statistically significant difference from the control group ($p < 0.05$, Dunnett's multiple comparison).

Fig. 1. Photomicrographs of cross-sections of a testis stained with hematoxylin and eosin. The pictures (A), (B) and (C) represent control, EGME low-dose (50 mg/kg) and high-dose (2,000 mg/kg) groups at 24 hr after the administration, respectively. Degenerative changes of spermatocytes were observed with the high dose of EGME (black arrows).

along the length of the epididymis. The caput region of the epididymis contained a large amount of lipids, such as triglycerides and fatty acids (Fig. 2B), while the cauda region of the epididymis had a high content of phosphocholine and glycerophosphocholine (Fig. 2C).

Metabonomic analysis of reproductive organs

We investigated the effect of EGME on reproduc-

tive organs using Hr-MAS ^1H -NMR spectrometric analysis. Fig. 3 shows the changes of metabolic profiles affected by EGME treatment both in testis and caput epididymis. The initial PCA analysis of the Hr-MAS ^1H -NMR spectra of reproductive organs showed the effects of EGME on each reproductive organ. The PCA scores plot of testis spectra revealed apparent differences among the three groups of treatment, indicat-

Fig. 2. Typical one-dimensional Hr-MAS ^1H -NMR spectra (600 MHz) of intact reproductive tissues obtained from a control rat: testis (A), caput region of epididymis (B), and cauda region of epididymis (C). These reproductive tissues were obtained from the same animal. Spectra were acquired using a CPMG-presaturation pulse sequence at 5,000 Hz spinning rate (total spin-echo relaxation delay: 60 ms).

Keys: 1, lipid (triglycerides, fatty acids); 2, leucine, isoleucine; 3, lactate; 4, alanine; 5, lysine; 6, acetate; 7, glutamate; 8, glutamine; 9, glutathione; 10, citrate; 11, creatine; 12, choline; 13, phosphocholine, glycerophosphocholine; 14, phosphatidylcholine; 15, myo-inositol; 16, creatinine; 17, *N*-methyl nicotinate; 18, α -glucose.

Metabonomic investigation of testicular toxicity induced by EGME.

ing that EGME-related changes occurred in the testes at the level of endogenous metabolites (Fig. 4a). Regarding the caput region of the epididymis, clusters of the calculated PCA scores in the treated groups were

relatively separated from those in the control group (Fig. 4b). The PCA analysis of the cauda epididymis did not show treatment-related alterations at the level of endogenous metabolites (Fig. 4c).

testis caput epididymis

Fig. 3. Series of 600 MHz ^1H -MAS ^1H -NMR spectra (δ 0.5-4.5) of testis and caput epididymis from control rat and rats dosed with EGME (low- and high-dose) obtained at 24 hr post dose.

Fig. 4. Unsupervised PCA scores plots of reproductive organs excised from rats at 24 hr after EGME administration. PCA scores plots for the first 2 principal components ($t[1]$, $t[2]$) of testes (a), caput region of the epididymides (b), and cauda region of the epididymides (c), showing clustering according to the damage induced by EGME on tissues. PCA scores were calculated on the basis of Hr-MAS ^1H -NMR CPMG spectra. The closed diamonds and open and closed triangles represent the control, and low- and high-dose EGME groups, respectively. The confidence interval is defined by the Hotelling's T^2 ellipse (95% confidence interval). Several observations were omitted in PCA scores plots of testes (a) and cauda epididymides (c), because the observations were plotted outside the confidence ellipse and considered to be outliers.

The changes in endogenous metabolites in the testes and caput epididymis are summarized in Table 3. They were disclosed by subsequent PLS-DA analysis. The levels of 11 endogenous metabolites in the testes and 1 in the caput epididymides showed a decrease or increase after the administration of EGME. The pre-

dominant changes were an increase of lactate, acetate and amino acids, and a decrease of creatine, glutathione (GSH) and phosphocholine. The level of lipid decreased in the caput region of the epididymis.

Fig. 5. Unsupervised PCA plots of scores for the first 2 principal components ($t[1]$, $t[2]$) based on ^1H -NMR spectra of urine (a) and plasma (b) of rats administered EGME. The symbols, which are colored with gray, white and black, represent pre-dose (or control), and low and high dose of EGME, respectively. As for the PCA scores plot of urine (a), the triangles and squares indicate 0 to 8 hr and 8 to 24 hr collection periods, respectively. Regarding the PCA scores plot of plasma (b), the triangles and squares indicate the 6 hr and 24 hr time points, respectively.

Metabonomic analysis of biofluids

The ^1H -NMR spectra of urine and plasma obtained at two time points and two dose levels exhibited an altered biochemical composition. The PCA scores plot of urine ^1H -NMR spectra showed that the three different clusters, non-affected (pre-dose and low-dose) and affected (high-dose), were clearly organized (Fig. 5a). The PCA scores of plasma at control and high-dose were relatively separated (Fig. 5b).

Table 3 also shows the affected endogenous metabolites in urine and plasma as well as in reproductive organs. In total, 8 metabolites were affected in urine and 5 in plasma. The major biochemical changes identified in urine were a decrease in the levels of 2-oxoglutarate, citrate, hippurate and succinate, which are intermediates in the tricarboxylic acid (TCA) cycle. Moreover, it was found that ketone-bodies, acetoacetate and α -hydroxyisovalerate, were increased in urine. In plasma, an increase in alanine and lactate, a decrease in α -glucose, β -hydroxybutyrate and glycine were detected.

DISCUSSION

“Solid-state” Hr-MAS ^1H -NMR spectroscopy enables the assessment of tissues in the intact *ex vivo* state, and also the investigation of the content of

endogenous metabolites in the cells. The use of Hr-MAS ^1H -NMR to evaluate reproductive organs was first introduced by Griffin *et al.* in 2000. They performed a Hr-MAS ^1H -NMR analysis of testes and then catalogued the major metabolites they found by peak assignment. Here we report the first Hr-MAS ^1H -NMR spectra of rat epididymides (both caput and cauda regions) as well as testis, and their usefulness to evaluate testicular toxicity induced by a male reproductive toxicant. The testicular spectrum shown in Fig. 2A was identical to previously reported spectra. It was known that the lipid content of epididymal spermatozoa of rats changes along the epididymis (Adams and Johnson, 1977). Lipid changes occur during the epididymal transit of spermatozoa as they mature, indicating that alterations in membrane fluidity by lipid remodeling plays an important role in determining the structure and functions of the sperm membrane (Rana *et al.*, 1991; Avelandano *et al.*, 1992). According to our finding from the epididymal Hr-MAS ^1H -NMR shown in Figs. 2B and 2C, the lipid content in the caput epididymis was higher than that in the cauda, which was consistent with data from previous reports.

In the metabonomic analyses of reproductive organs, the score plots from PCA and PLS-DA analyses of testicular Hr-MAS ^1H -NMR spectra demonstrated that each treatment group formed different clus-

Table 3. Summary of endogenous metabolites detected by ^1H -NMR metabonomics that were induced or suppressed by EGME in reproductive organs and biofluids of male rats.

		Affected Endogenous Metabolites	
		Increase	Decrease
Tissues	Testis	- acetate (high) - alanine (low<high) - glutamate (high) - isoleucine (high) - lactate (low<high) - leucine (high) - lysine (low<high)	- creatine (high) - phosphatidylcholine (high) - phosphocholine (low=high) - glutathione (low=high)
	Epididymis (caput region)		- lipid (low=high)
Biofluids	Urine	- acetoacetate (high-8<high-24) - α -glucose (high-8>high-24) - α -hydroxyisovalerate (high-8=high-24) - β -hydroxybutyrate (high-8>high-24)	- 2-oxoglutarate (high-8<high-24) - citrate (high-8<high-24) - hippurate (high-8>high-24) - succinate (high)
	Plasma	- α -glucose (high-6=high-24) - β -hydroxybutyrate (high-24) - glycine (high-6>high-24)	- alanine (high-6=high-24) - lactate (high-6=high-24)

Variations compared to control samples.

Treatment and time point at which the changes in the level of endogenous metabolites were detected are shown in parentheses, namely, (high-8) means that the change was observed at 8 hr after the administration of a high dose of EGME.

ters in scores plots (Fig. 4a), indicating that EGME was highly affected to endogenous metabolites. In the testes, the major alterations were an increase in lactate and decreases in creatine and GSH. In the testes and the caput epididymides, the components of the sperm membrane, such as phosphocholine, phosphatidylcholine and lipid, were also mainly affected.

Testicular lactate increased and plasma lactate decreased in the present study. Lactate is produced via pyruvate metabolism from glucose subsequent to cytosolic glycolysis, which occurs in Sertoli cells (Robinson and Fritz, 1981; Bajpai *et al.*, 1998). It is considered that testicular organs need great energy to continuously maintain spermatogenesis. It was reported that intracellular infusion of lactate improved spermatogenesis by preventing loss of germ cells in the rat cryptorchid testes (Courten and Ploen, 1999). Erkkila *et al.* (2002) also demonstrated that lactate effectively inhibited apoptosis of testicular germ cells in a dose-dependent manner. As described above, lactate is an essential substrate in spermatogenesis for the regulation of survival of pachytene spermatocytes (Jutte *et al.*, 1981, 1982). Lactate seems to be supplied in the seminiferous tubules from two main sources, an endogenous (intracellular) and an exogenous (extracellular) one (Brauchi *et al.*, 2005). One of these sources (routes) is the supply from Sertoli cells, and this is mediated by the L-lactate transport system with monocarboxylate transporter 4 (MCT4) in spermatogenic germ cells. On another route, lactate is also uptaken by pachytene spermatocytes from blood using MCT1. EGME is a specific toxicant to spermatocytes, but methoxyacetic acid (MAA), which is the major metabolite of EGME, also inhibits lactate production in Sertoli cells *in vitro* (Beattie *et al.*, 1984). Under normal conditions, the intracellular concentration of lactate is regulated by hormonal control and supply. However, this balance is perturbed when the testes are toxicologically damaged. The alterations of lactate content detected in the present study might be attributed to a perturbation of this balance due to spermatocytes degeneration induced by EGME.

In the testes, creatine content decreased in the high-dose group. As shown by testicular H¹-MAS NMR spectroscopy, testicular tissue has high concentrations of creatine. In fact, creatine concentration in rat testes and seminal fluid is significantly higher than in blood (Lee *et al.*, 1988). The finding indicated that sperm cells require a high concentration of creatine to maintain their viability and function. Timbrell and co-workers reported that creatine in urine and plasma was

associated with testicular damage induced by EGME, MAA, cadmium, di-n-pentyl phthalate (DPP), and 1,3-dinitrobenzene (1,3-DNB) (Rawcliffe *et al.*, 1989; Gray *et al.*, 1990; Moore *et al.*, 1992; Timbrell, 2000). They concluded that creatine might serve as a useful non-invasive biomarker of male reproductive dysfunctions. In the present metabonomic study, however, urinary and plasmatic creatine did not change. This was probably because the spermatocytes degeneration induced by EGME was relatively mild, as demonstrated by conventional toxicity data showing no changes in reproductive organs weight and minor histopathological findings. According to the investigations conducted by Timbrell *et al.*, the elevation of creatine in urine was likely to be caused by severe testicular damage. Therefore, our results are consistent with previous findings.

As another considerable mechanism of testicular toxicity, the effect of oxidative stress on testicular toxicity should be considered. The significant reduction in GSH level in mouse or rat testes has been previously documented (Shen and Sangiah, 1995; Turk *et al.*, 2007). In the present study, testicular GSH also decreased after the administration of EGME. Consequently, oxidative stress was thought to contribute to the testicular toxicity induced by chemicals. In addition, alterations in the expression of oxidative stress-related genes and proteins would further support this mechanism. We have already documented that the expression of antioxidant protein 2 and glutathione S-transferase at both the protein and transcription levels is altered by reproductive toxicants (Fukushima *et al.*, 2005; Yamamoto *et al.*, 2005).

The urine metabonomic analysis showed decreased excretions of the TCA cycle intermediates, 2-oxoglutarate, citrate and succinate. Additionally, the ketone-bodies, acetoacetate and β -hydroxybutyrate, were increased in urine. Normally, ketone-bodies are transported from the liver to other tissues including reproductive organs, where they can be reconverted to acetyl-CoA to produce energy. An impairment of the TCA cycle leads to increased release of ketone-bodies from liver for use as fuel in other tissues. When evaluating metabonomic changes in biofluids such as plasma and urine, we should pay much attention to the specificity of damaged organs in the body. Indeed, the alterations of TCA cycle intermediates in the present study might offer a general decrease of energy metabolism, which was supported by the decreased body weight in high-dose 24 hr group. In addition, we also attributed the decrease in urinary TCA cycle intermedi-

ates observed in this study to the suppression of TCA cycle in the testes, and the increase of ketone-bodies in urine and plasma might reflect that effect of EGME. Elevations of amino acids in the testes suggested that the TCA cycle was likely to be suppressed in testicular organs. As for the relevance of TCA cycle suppression in testicular toxicity, it was reported that spermatocytes and spermatids died when the TCA cycle was inhibited (Jutte *et al.*, 1982).

Based on the present testicular metabonomic analysis, we propose that testicular toxicity induced by EGME resulted from either perturbation of the energy supply processes, suppression of the TCA cycle, or oxidative stress, or all of these factors. In summary, integration of the analyses of intact tissues and biofluids using ¹H-NMR provides a more detailed knowledge of *in vivo* molecular events. Hr-MAS ¹H-NMR spectroscopy of intact tissues is useful in toxicological investigations because the spectra are relatively easy to obtain within a few minutes, and measurements are conducted in a high throughput manner. Using Hr-MAS ¹H-NMR, it is possible to investigate whether a target organ is normal or damaged, before histopathological changes can be detected.

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