Aerosol and Air Quality Research, 14: 1714–1725, 2014

ISSN: 1680-8584 print / 2071-1409 online
doi: 10.4209/aaqr.2013.07.0260

Metabolomic Analysis of the Effects of Motorcycle Exhaust on Rat Testes and Liver

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ABSTRACT

Motorcycles are one of the primary means of transportation in many Asian metropolitan areas. Exposure to motorcycle exhaust (ME) has been shown to cause male reproductive toxicity in a rat inhalation model. In the present study, the molecular mechanisms underlying ME-induced male reproductive toxicity were examined in a rat model using nuclear magnetic resonance (NMR)-based metabolomics. Rats were treated with 1:10-diluted ME for 1 hour in the morning and 1 hour in the afternoon, daily, via head-nose-only exposure chambers, for a period of four weeks. Half of the ME-exposed rats were co-treated with vitamin E to examine its protective effects. Both hydrophilic and hydrophobic metabolites from the testes and liver were extracted for ^{1}H and J-resolved NMR analyses. The obtained NMR spectra were converted into series of spectral binned areas and subjected to orthogonal projections to latent structure-discriminant analyses. Decreased spermatid counts and elevated levels of betaine in the testes of rats in the ME-treated group were partially attenuated by vitamin E co-treatment. Although it was not statistically significant, we observed a consistent trend of partial reversal of the elevated levels of alanine, glycine, leucine, tyrosine, and valine in the testes of rats in the ME-treated group due to vitamin E co-treatment. Decreases in testicular ethanolamine and inositol were only significant in the group co-treated with ME and vitamin E. The testicular toxicity of ME may be due to alterations in lipid- and energy-related metabolism. We conclude that ME generates greater metabolic responses in the testes than in the liver. Treatment with vitamin E can partially reverse the molecular events in the testes.

Keywords: Motorcycle exhaust; Metabolomics; Male reproductive toxicity; Nuclear magnetic resonance; Testes.

ABBREVIATIONS

NMR (nuclear magnetic resonance); OPLS-DA (orthogonal projections to latent structure-discriminant analysis); ME (motorcycle exhaust); PAH (polycyclic aromatic hydrocarbon); MEP (motorcycle exhaust particulates); CYP (cytochrome P450); IL (interleukin); FGF (fibroblast growth factor); Vit (vitamin); JRES (J-resolved); TMSP (3-trimethylsilyl-2,2,3,3-d_4-propionate); TMS (tetramethylsilane); *p*-JRES (skyline projection J-resolved); ANOVA (analysis of variance); PE (phosphatidylethanolamine); PC (phosphatidylcholine).

INTRODUCTION

Motorcycles are one of the most commonly used transportation tools in Asia due to their convenience and mobility and the ease of owning, using, and parking them. There are more than 13 million registered motorcycles, which amounts to one vehicle for every two people in Taiwan. The vehicle density (ca. 466 vehicles/km^2) in Taiwan is nearly the highest in the world (R.O.C., 2012). Motorcycle engines, especially 2-stroke engines, are characterized by incomplete gasoline combustion; therefore, their exhaust presents a significantly greater contribution to air pollution than the

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exhaust from 4-stroke motorcycles and passenger cars (Vasic and Weilenmann, 2006). To date, the air pollution and health effects caused by motorcycle exhaust (ME) have been largely underestimated and ignored. Information regarding the potential health risks due to ME is not readily available.

ME may contain gas, volatile organic compounds, semi-volatile organic compounds, and particulates. Benzene, toluene, ethyl benzene, xylene, polycyclic aromatic hydrocarbons (PAHs), 1,3-butadiene, and other compounds have been found in the gas phase of ME (Chang and Chen, 2008). Both noncarcinogenic and carcinogenic PAHs, such as naphthalene, benzo[a]pyrene, benz[a]anthracene, and chrysene, were detected in ME particulates (MEP) (Ueng *et al.*, 2005; Yang *et al.*, 2005b; Chang and Chen, 2008). Nearly 90% of the particulates and PAH mass from the exhaust of 2-stroke motorcycles had diameters smaller than 0.25 μm (Yang *et al.*, 2005a); therefore, these particulates and compounds can travel deep into the lungs and enter the whole body via the circulation system.

MEP and MEP extracts have been demonstrated to be mutagenic (Zhou and Ye, 1997), genotoxic (Kuo *et al.*, 1998), and anti-estrogenic (Ueng *et al.*, 2004a) *in vitro*. The effects of ME on the respiratory system have been studied both *in vitro* and *in vivo* using cDNA microarray analysis (Ueng *et al.*, 2005). MEP resulted in increases in the mRNA levels of cytochrome P450 (CYP) 1A1 and 1B1, cytokines interleukin (IL)-1(α), IL-6, and IL-11, fibroblast growth factor (FGF)-6 and FGF-9 in human adenocarcinoma cells. Increases in the CYP 1A1, IL-1(α), and FGF-9 mRNA levels were also detected in the lungs of rats that had inhaled ME (Ueng *et al.*, 2005).

Moreover, animal models demonstrated that ME causes neurotoxicity, immunotoxicity (Lee *et al.*, 2004), anti-estrogenicity (Ueng *et al.*, 2004a), and male reproductive toxicity (Huang *et al.*, 2008). ME inhalation elevated CYP 1A1 protein levels and monooxygenase activity (Ueng *et al.*, 1998; Ueng *et al.*, 2004b) in the rat liver, lungs, and kidneys. Alterations in CYP 2B1 protein levels were found to be organ specific (Ueng *et al.*, 2004b). Several antioxidant enzymes, such as superoxide dismutase and catalase, were decreased in the hepatocyte cytosol as well. ME also increased susceptibility to lipid peroxidation based on a thiobarbituric acid assay in the livers and lungs of rats. In addition, the IL-6 mRNA and protein levels were elevated in the testes of these same rats (Huang *et al.*, 2008). The results of these studies suggested that oxidative stress is one of the mechanisms underlying ME-mediated toxicity both *in vitro* and *in vivo*.

The results of a recent study highlighted the adverse effects of ME on the male reproductive system using a rat inhalation model (Huang *et al.*, 2008). Reduced testicular weights, spermatid numbers, cauda epididymal sperm numbers, and male mating indices were observed in rats exposed to ME twice daily for a period of 4 weeks. ME caused in a trend toward decrease of glutathione level and increase of lipid peroxidation in the rat testes. Decreased enzyme activities of 7-ethoxycoumarin *O*-deethylase and superoxide dismutase in the testes were also related to ME exposure. Moreover, the adverse effects of ME, including decreased testosterone and testicular spermatid number, and increased IL-6 mRNA and protein levels, can be partially reversed by vitamin (Vit) E co-treatment, suggesting that ME-induced male reproductive toxicity may be due in part to oxidative stress and the inhibition of steroidogenesis.

Metabolites represent the downstream biochemistry that is directly involved in biological regulation and are therefore closely correlated with phenotype. Furthermore, the metabolome can reflect the physiological conditions in a biological system that is altered by environmental stress or genetic modifications. As with other "-omics" techniques, metabolomics is high-throughput and unbiased. It does not focus on particular metabolic groups based on chemical structure or biological function. Therefore, an overview of the metabolic status that reflects an organism's phenotype can be described. The metabolomic approach has been widely applied in the fields of clinical pharmacology and toxicology, as well as environmental toxicology (Lin *et al.*, 2009a; Santos *et al.*, 2009). Understanding the impact of xenobiotics or environmental stress on the metabolome will help researchers understand the mechanisms underlying toxicity and will assist in further biomarker development.

In the present study, we assessed the metabolic responses of the male reproductive system to ME exposure and compared them to those of the liver, a major detoxifying organ, using a rat inhalation model. Vit E co-treatment was used to further assess whether oxidative stress is the major toxic mode of action caused by ME. A nuclear magnetic resonance (NMR)-based metabolomic approach was applied to examine the metabolic responses and to further investigate the possible mechanisms underlying ME-induced male reproductive dysfunction.

MATERIALS AND METHODS

Animals and Experimental Design

Male Wistar rats (7 weeks of age) were obtained from the Animal Center of the College of Medicine at National Taiwan University. The animals were housed in an animal room with air conditioning and 12-h light/dark cycles for one week prior to conducting the experiments. The animals were given access to water and rodent laboratory chow (Purina Mills, Inc., St Louis, MO). Twenty-one animals were randomly assigned to 1 of 3 groups: an ME treatment group (n = 6), an ME and Vit E co-treatment group (n = 8), and a control group (which was treated with clean air; n = 7).

For the Vit E co-treatment group, ME-exposed rats were orally administered 50 mg/kg/day of (+)-α-tocopherol (100 IU/g, Sigma-Aldrich Corporation, St. Louis, MO), Monday through Friday, for a period of 4 weeks. The control and ME-treated groups were subjected to gavage with corn oil, vehicle of Vit E. The control and ME-treated groups were handled in the same manner as the ME + Vit E-treated group. After the final exposure, the animals were sacrificed by decapitation under light anesthesia with CO_2. The use and care of these animals was approved by the Institutional Care and Use Committee, College of Medicine, National Taiwan University.

Exposure Systems

A 1992 Yamaha Cabin motorcycle equipped with a 2-stroke 50-cc engine and a variable venture carburetor was used to generate ME. The vehicle had a mileage of 6658 but functioned well and met the emission standards of the Environmental Protection Administration of Taiwan. The exposure system conditions have been described previously (Ueng *et al.*, 2004a). In brief, the vehicle was fueled with unleaded gasoline and run on idle speed with an empty load to simulate a motorcyclist sitting in traffic or waiting for traffic lights to change in urban areas. The ME was delivered to the chambers, dehydrated, and diluted 1:10 using filtered air. The diluted ME was then delivered to a head-nose-only exposure inhalation chamber (Technical & Scientific Equipment GMBH, Bad Hamburg, Germany) for rat inhalation. The diluted ME was brought to the inner concentric cylinder in the chamber and delivered to the periphery of the outer cylinder, which contained 21 openings for rat exposure holders and 2 exposure ports for air sampling. The animals were exposed to the diluted ME from 9 to 10 am and 4 to 5 pm daily, Monday through Friday, for a period of 4 weeks.

The exposure chamber atmospheres were analyzed using an aerosol monitor DustTrak 8520 (TSI, Inc.), with a cutoff at 10 μm, and a CA-6200 combustion analyzer (TSI, Inc.). The mean concentrations of exposure chamber atmospheres in the control and ME exposure groups were as follows: particles, 0.4 and 21.4 mg/m^3; oxygen, 20.4 and 20.3%; carbon monoxide, 0 and 475.9 ppm; carbon dioxide, 0 and 3.7%; nitric oxide, 0 and 3.9 ppm; and nitric dioxide, 0 and 3.7 ppm, respectively. The measurement ranges for particles, oxygen, carbon monoxide, carbon dioxide, nitric oxide, and nitric dioxide are 0.001 to 100 mg/m^3, 0–25%, 0–20,000 ppm, 0-CO_2 Max, 0–4,000 ppm, and 0–500 ppm, respectively. The composition of the ME particles and organic extracts of the ME particulate were determined by Ueng *et al.* (2005) and were as follows: the mean concentrations of PM_1, $PM_{2.5}$ and PM_{10} were 118 mg/m^3, 216 mg/m^3, and 228 mg/m^3, respectively. The composition and mean concentrations of PAHs in the organic extract of the ME particulates are summarized in the Supplementary Materials (Table S1).

Spermatid and Sperm Number Determinations

The testes were homogenized in phosphate-buffered saline (pH 7.4) using a Teflon-pestle homogenizer. Spermatid numbers were counted from the diluted sperm suspensions on a hemocytometer using a phase-contrast microscope. The cauda epididymal sperm numbers were determined in a similar way.

Metabolite Extraction from Tissue Samples for Metabolomic Analyses

The metabolite extraction method was mainly adapted from that used in the previous studies (Lin *et al.*, 2007). Briefly, the frozen tissue (hepatic and testicular) samples were ground using a mortar and pestle in liquid nitrogen. The tissue powders were then dried using a lyophilizer, and the dried tissues (~20 mg) were then extracted using chloroform/methanol/water at a final ratio of 2:2:1.8 (Bligh and Dyer, 1959; Lin *et al.*, 2007). Following centrifugation at 10,000 g and 4°C for 15 min, both the upper (hydrophilic) and lower (hydrophobic) layers were carefully removed and dried. The dried metabolite extracts were stored at –80°C prior to NMR analysis.

^{1}H and J-resolved NMR Spectroscopy

Both ^{1}H and J-resolved (JRES) NMR spectra were obtained using AVANCE 500 AV (for the hepatic extracts) and AVANCE 600 AV (for the testicular extracts) spectrometers (Bruker, Germany) equipped with cryoprobes. The parameters of the NMR acquisition and spectral processes have been described previously (Viant *et al.*, 2003; Beckonert *et al.*, 2007; Lin *et al.*, 2007). The hydrophilic metabolites were resuspended in D_2O containing 0.1 M sodium phosphate buffer (pH 7.4) and 3-trimethylsilyl-2,2,3,3-d_4-propionate (TMSP, a chemical shift standard). The hydrophobic extracts were resuspended in d-chloroform containing 3% (vol/vol) tetramethylsilane (TMS). The ^{1}H NMR acquisition parameters included an 8-μs (60°) pulse, a 6-kHz spectral width, a 2.5-s relaxation delay, and a temperature of 300 K. The residual water resonance from the hydrophilic extract samples was suppressed by presaturation. The ^{1}H NMR spectra were phased, baseline corrected, and calibrated using TMSP or TMS with Topspin software (Version 2.1, Bruker, Germany).

JRES NMR spectra were obtained using 4 transients per increment, for a total of 32 increments. Sixteen data points, using spectral widths of 40 Hz in F1 (spin-spin coupling constant axis) and 6 kHz in F2 (chemical shift axis), were collected. The datasets in F1 were zero-filled to 128 points, and both dimensions were multiplied by sine-bell window functions following Fourier transformation. The spectral process parameters included a tilt, F1 symmetrization, TMSP or TMS calibration (0 ppm), and proton-decoupled skyline projections (*p*-JRES), all using Topspin.

Peaks assignments in both the ^{1}H and *p*-JRES NMR spectra were confirmed using tabular data found in the previously published literature (Casu *et al.*, 1991; Fan, 1996; Tugnoli *et al.*, 2003; Oostendorp *et al.*, 2006; Lin *et al.*, 2009a, b; Ong *et al.*, 2009) and the Chenomx NMR software suite (Professional Edition, Version 4.6, Chenomx, Inc.).

p-JRES NMR Spectral Processes for Statistical Analyses

p-JRES NMR spectra were binned between 0.2 and 10.0 ppm at a bin width of 0.005 ppm to form a total of 1960 segments using a customer-written program, *ProMetab* (Version 2.1, (Viant, 2003)), linked with MATLAB (Version 7.8, The MathWorks, Natick, MA). *ProMetab* further integrated the area within each spectral segment and generated a 1 × 1960 vector to describe the original spectrum. Spectral bins corresponding to residual water resonances were removed. In some cases, the bins were compressed to a single bin to capture peaks with slightly different chemical shifts. The total spectral area of the remaining segments was normalized to unity to facilitate comparisons between the spectra. The binned data were then subjected to log transformation, and the variables were mean-centered prior to conducting the multivariate analyses.

Statistical Analyses

Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) were applied in this study. PLS is a latent variable regression method that is based on the assumption that X (metabolites) can be described by fewer latent variables. In PLS, latent variables are calculated to maximize the co-variation between X and Y (treatment classes). O-PLS further analyzes the variation in each PLS component and removes non-correlated systemic information (orthogonal variation to Y) in X (Trygg and Wold, 2002). Therefore, O-PLS improves the interpretational ability and reduces model complexity. OPLS-DA has been applied when modeling classes of data to maximize class separation, simplify interpretation, and identify potential biomarkers.

Spectral data were imported to SIMCA-P+12 (Umetrics, Umea, Sweden) for the multivariate statistical analysis. OPLS-DA models were validated using a 7-round cross-validation procedure. Cross-validation can provide information that can be used to determine the amounts of significant components by analyzing the total amounts of explained X (R^2X) and Y (R^2Y), and the cross-validated predictive ability (Q^2). R^2Y and Q^2 represent how well the model fits the Y and the predictive accuracy of the model, respectively. Then, loading plots and S-plots illustrate metabolites that contribute to the class separation.

The spectral bins contributing to the clusters were identified, and the changes in the corresponding peak areas among the experimental groups were further examined using analysis of variance (ANOVA) followed by Duncan's new multiple range test.

RESULTS

Effects of ME Treatment Alone and ME Plus Vit E Co-treatment on Body Weight, Organ Weight, and Sperm Count In Rats

ME inhalation, with or without Vit E co-treatment, resulted in a 12% decrease in body weight (Table 1). ME inhalation decreased the testicular weight, testicular and cauda epididymal spermatid count by 19%, 54%, and 76%, respectively. However, co-treatment with Vit E lessened the effects of ME inhalation, resulting in a 12% decrease in testicular weight, a 29% decrease in testicular spermatid count, and a 66% decrease in cauda epididymal spermatid count compared with the control group. The ME and ME + Vit E groups showed significantly reduced testicular weight, testicular spermatid count, and cauda epididymal sperm count compared to the control group ($p < 0.01$). Although a trend toward increased sperm count was observed in the testes and cauda epididymides after Vit E co-treatment, it was only statistically significant in the cauda epididymides ($p < 0.05$).

NMR Spectra and Metabolite Assignment

The representative ^{1}H and *p*-JRES NMR spectra of hydrophilic and hydrophobic metabolites from the testes and liver are shown in Figs. 1–2 and Supplemental Figs. S1–S2, respectively. The ^{1}H and *p* -JRES NMR spectra of the hydrophilic extracts were similar, with the exception of the *p*-JRES generated simple and well-resolved peaks with a flat baseline. Unlike the similarities observed between the ^{1}H and *p*-JRES NMR spectra of the hydrophilic extracts, several hydrophobic metabolites (i.e., fatty acyl chains –C**H**$_2$CH= and =CHC**H**$_2$CH=) displayed stronger signals in the ^{1}H NMR spectra, while a few of the metabolites (i.e., triglycerides) displayed stronger peaks in the *p*-JRES NMR spectra. The peak location (ppm), multiplicity, and integrated areas in both the ^{1}H and *p*-JRES NMR spectra provided information for peak assignment. The likelihood of observing and correctly identifying metabolites increased by examining both spectra. Only the peaks that satisfied all of the criteria were assigned (Supplemental Tables S2 and S3). The *p*-JRES spectra provided flat baselines with simple and well-resolved peaks, as described earlier (Viant, 2003; Ludwig and Viant, 2010); therefore, these spectra were further processed to generate binned spectral areas for multivariate analyses.

Metabolic Responses to ME Treatment Alone and ME Plus Vit E Co-treatment in the Testes

p-JRES NMR spectra of both the hydrophilic and hydrophobic metabolites from the testes and liver were further analyzed using OPLS-DA. OPLS-DA scores plots can demonstrate the groupings, trends, and outliers of observations (samples). Within the scores plots, each symbol represents one sample, and the closer these symbols are to each other, the closer their properties are. Two outliers (1 control and 1 ME sample) that fell outside of Hotelling's T^2 distribution were observed in the scores plot from the analyses of testicular hydrophilic metabolite spectra and were therefore excluded. The final OPLS-DA model (2 predictive components) was established based on the metabolome of

Table 1. Effects of motorcycle exhaust (ME) treatment alone and ME plus vitamin E co-treatment on body weight, organ weights and sperm count in rats.

	Control	ME	ME + Vitamin E
Body weight (g)	351.66 ± 10.12^{a}	309.61 ± 7.43^{b}	309.8 ± 8.42^{b}
Liver weight (g)	12.23 ± 0.375^{a}	10.90 ± 0.683^{a}	11.53 ± 0.580^{a}
Left testis weight (g)	1.878 ± 0.029^{A}	1.506 ± 0.090^{B}	1.661 ± 0.044^{B}
Right testis weight (g)	1.832 ± 0.024^{A}	1.509 ± 0.096^{B}	1.616 ± 0.046^{B}
Testicular spermatid density (spermatid/mg testis × 10^3)	32.9 ± 4.7^{A}	15.2 ± 2.8^{B}	23.3 ± 3.1^{B}
Cauda epididymal sperm count (spermatid/mg cauda epididymis × 10^3)	544.5 ± 24.3^{Aa}	131.7 ± 16.5^{Bb}	185.0 ± 17.8^{Bc}

Each value represents the mean ± SE. Different letters following the mean in each column represent statistical significance by Duncan's new multiple range test. Capital letters and lowercase letters denote $p < 0.01$ and $p < 0.05$, respectively.

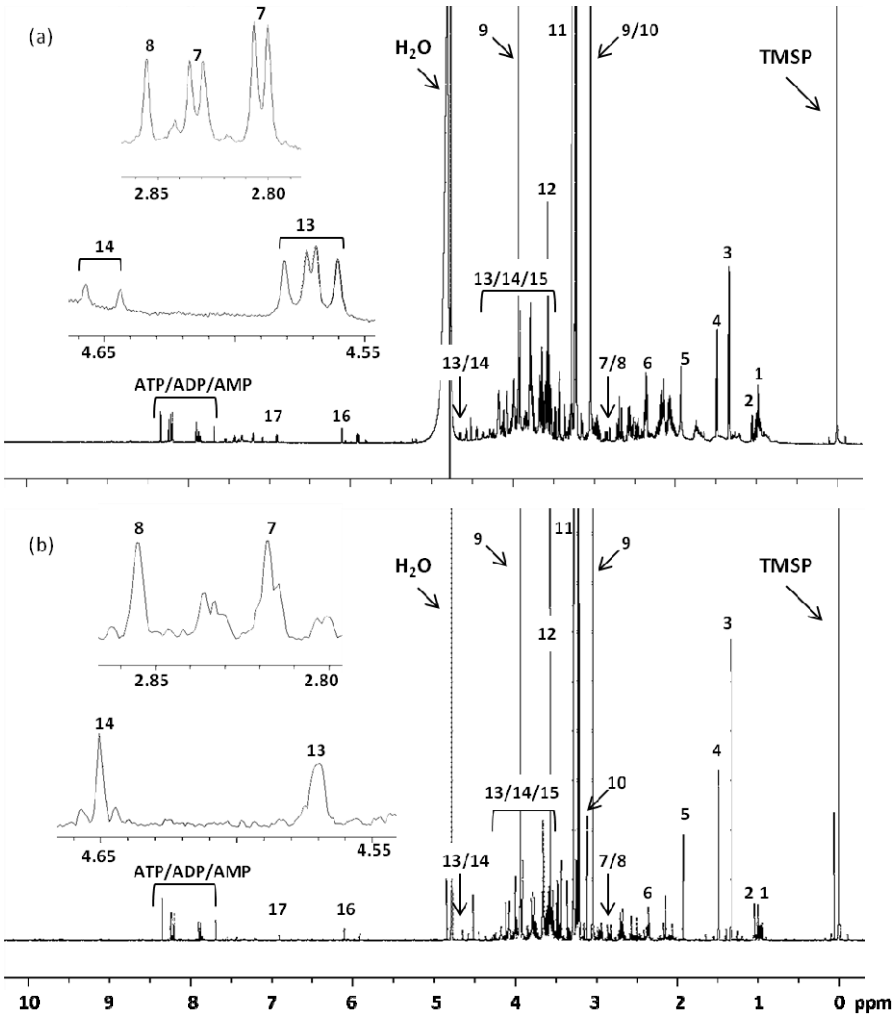

Fig. 1. Representative high-resolution (600 MHz) ^{1}H NMR (a) and *p*-JRES NMR (b) spectra for the hydrophilic metabolites extracted from the rat testes. The identified metabolites were as follows: 1. Leucine, 2. valine, 3. lactate, 4. alanine, 5. acetate, 6. glutamate, 7. aspartate, 8. trimethylamine, 9. creatine, 10. ethanolamine, 11. phosphocholine, 12. glycine, 13. glutathione, 14. glucose, 15. betaine, 16. adenosine, and 17. tyrosine.

the testicular hydrophilic metabolites from 3 groups (Fig. 3). The RX^2, R^2Y, and Q^2 were 0.896, 1.000, and 0.356, respectively. The OPLS-DA model explains the high variation between the groups. The components of ME in the group that was co-treated with Vit E were well separated from the other groups along the first predictive component. Furthermore, the second predictive component separated the ME-treated samples from the controls.

S-plots identify the variables (metabolites) that make the most important contributions to distinguish metabolic variation among groups in an OPLS-DA model. To solidify the significance of metabolic changes, the use of conventional statistical analytical methods, i.e., ANOVA, is essential. ANOVA, followed by Dunnett's test, in combination with S-plots (Figs. 4(a) and 4(b)) led to the identification of metabolites related to the clustering of hydrophilic metabolite spectra from the testes (Fig. 3). The levels of ethanolamine and inositol in the ME + Vit E-treated group were significantly lower than those in the control group ($p < 0.05$) (Fig. 5(a)). Moreover, betaine levels were significantly higher in the ME-treated group than that in the control group ($p < 0.05$). The levels of numerous amino acids, such as alanine, glycine, leucine, tyrosine, and valine, were elevated in the ME-treated group compared to the other two groups (Fig. 5(b)). The changes in these amino acids did not achieve statistical significance, although they were close to $p = 0.05$. Due to the small sample sizes in this study, the consistent increases in the levels of these amino acids that were observed in the ME-treated group may not have been random.

The OPLS-DA model of the testicular hydrophobic metabolite spectra did not cluster the samples based on treatment groups. However, a clear 2-group separation along the first orthogonal component was observed. Seven samples (2 control, 2 ME, and 3 ME + Vit E) were separated from the others. By comparing the original NMR spectra, we found that the spectra of those 7 samples contained series of peaks approximately 4.2, 7.5, and 7.7 ppm, which were assigned as lipid hydroperoxide/conjugated dienes (Waters *et al.*, 2002; Pajunen *et al.*, 2008). The lipid hydroperoxide

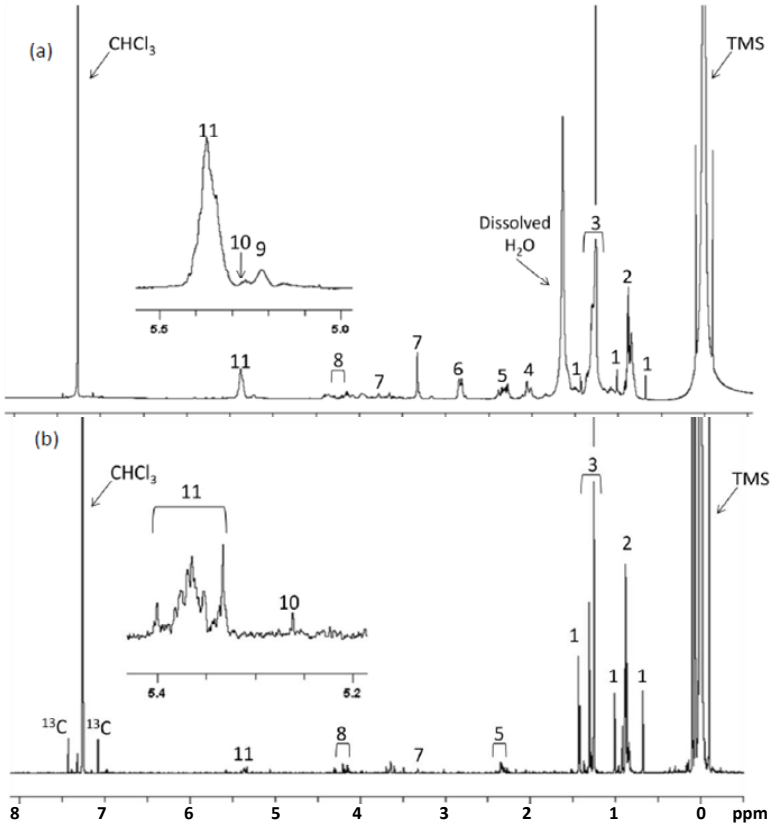

Fig. 2. Representative high-resolution (600 MHz) ^{1}H NMR (a) and *p*-JRES NMR (b) spectra from the hydrophobic metabolites extracted from the rat testes. The identified metabolites were identified as follows: 1. Cholesterol, 2. fatty acyl chain terminal C**H**$_3$, 3. fatty acyl chain (C**H**$_2$)$_n$, 4. fatty acyl chain –C**H**$_2$CH=, 5. fatty acyl chain –C**H**$_2$-CO, 6. fatty acyl chain =CHC**H**$_2$CH=, 7. phosphatidylcholine, 8. glycerol backbone C-1 **H**$_2$/C-3 **H**$_2$, 9. phospholipid glycerol backbone C-2 **H**$_2$, 10. triglyceride glycerol backbone C-2 **H**$_2$, and 11. fatty acyl chain –**H**C=C**H**-. The peaks labeled ^{13}C are the carbon-13 satellites of $CHCl_3$.

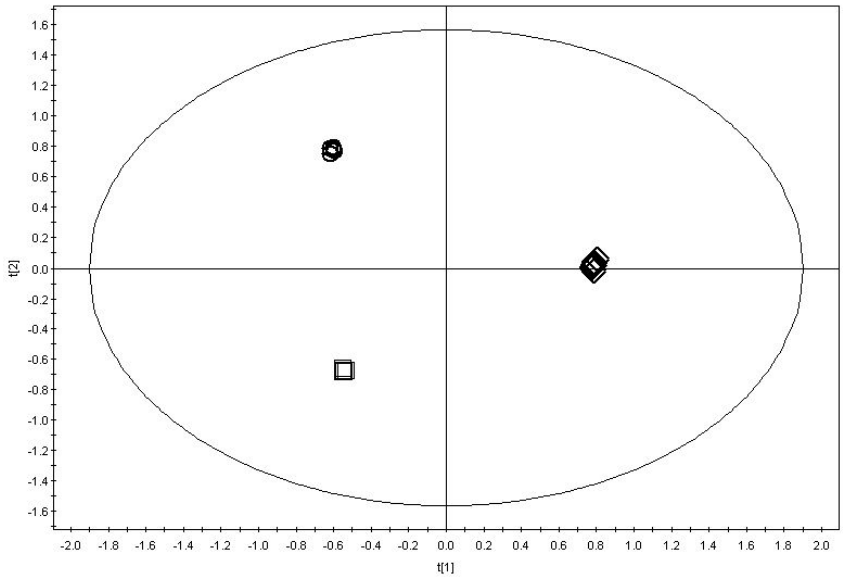

Fig. 3. OPLS-DA scores plot from the analysis of the p-JRES NMR spectra of the hydrophilic metabolites extracted from the testes of rats exposed to motorcycle exhaust (ME) (n = 5) (○), ME and vitamin E co-treatment (n = 8) (◇), or clean air (n = 6) (□). This pattern recognition model illustrates clear metabolic clustering among the 3 groups along the first two predictive components. Two outliers (1 from the control group and 1 from the motorcycle exhaust-treated group) that fell outside of Hotelling's T^2 distribution were excluded. The ellipse represents Hotelling's T^2 range (at a significance level of 0.05).

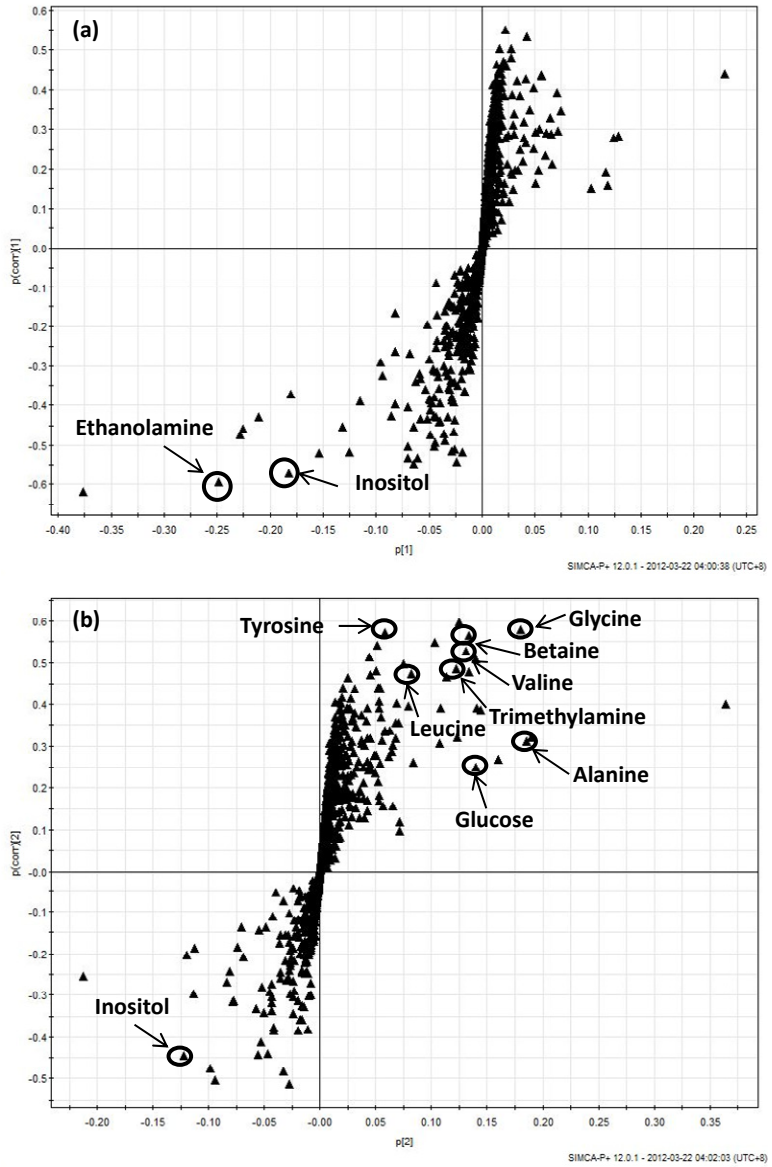

Fig. 4. S-plots corresponding to the first (a) and second (b) predictive components of the OPLS-DA model (Fig. 3) from the analysis of the *p*-JRES NMR spectra of the testicular hydrophilic extracts. Each spot denotes one variable that represents one spectral segment. Metabolites that made the most important contributions to distinguishing variations among the groups were identified.

reflected lipid peroxidation processes, which are closely related to cellular injury. However, these peroxide metabolites were not correlated with ME exposure.

Metabolic Responses to ME Treatment Alone and ME Plus Vit E Co-treatment in the Liver

The OPLS-DA models for the *p*-JRES spectra of the hydrophilic metabolites in the liver did not appear to be well separated among the 3 treatment groups. The R^2X, R^2Y, and Q^2 values were 0.526, 0.598, and 0.110, respectively. The majority of the well-resolved peaks from the *p*-JRES NMR spectra were identified (Supplemental Table S3) and selected to determine the significance of the changes using ANOVA; however, the metabolic changes were not statistically significant.

The OPLS-DA model of the *p*-JRES spectra of the hydrophobic metabolic extracts from the liver demonstrated little effects of ME or Vit E on the liver metabolome. The R^2X, R^2Y, and Q^2 values in this model were 0.324, 0.508, and 0.069, respectively. The majority of the well-resolved peaks in the NMR spectra were identified (Supplemental Table S3) and integrated following ANOVA to examine the equality among the groups. We found that the majority of the identified lipids were decreased following ME treatment, with or without Vit E treatment. Moreover, the increased levels of trimethylamine and decreased levels of phosphatidylcholine, unsaturated fatty acyl chains (-CH=CH-), and fatty acyl chain terminals ($-CH_3$) approached statistical significance (close or equal to p values of 0.05) (Fig. 6) in the ME + Vit E-treated group.

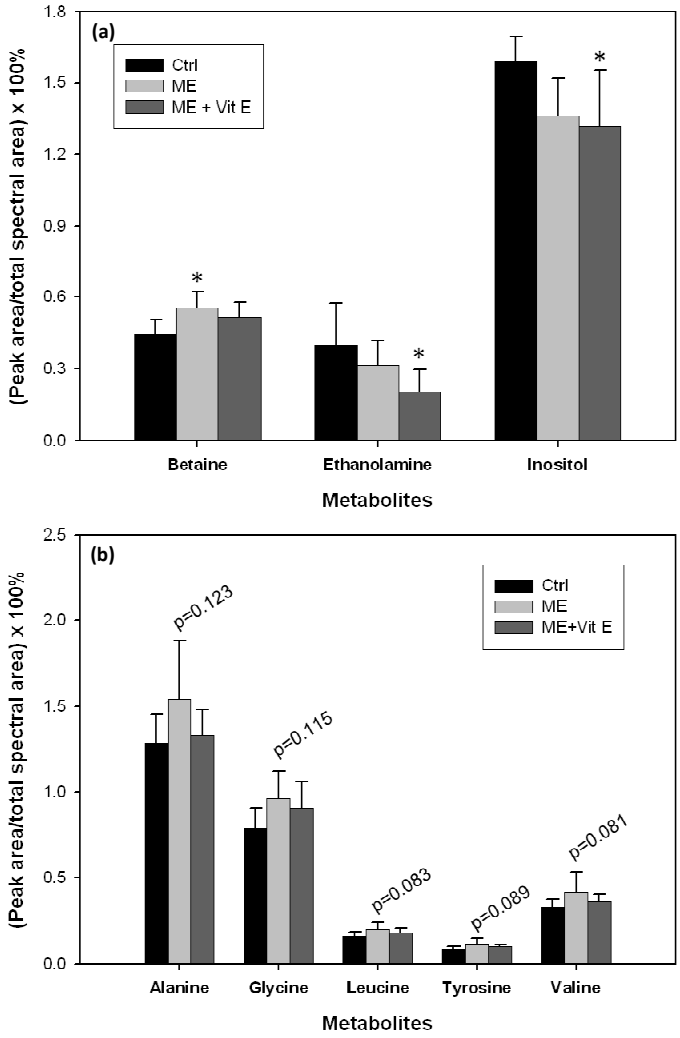

Fig. 5. Metabolic changes in the testes of rats exposed to motorcycle exhaust (ME), ME + Vitamin (Vit) E, or the control (Ctrl). (a) Significantly altered metabolites were highlighted (* denotes $p < 0.05$). (b) The levels of numerous amino acids were increased in the ME-treated group compared with the Vit E co-treatment and control groups.

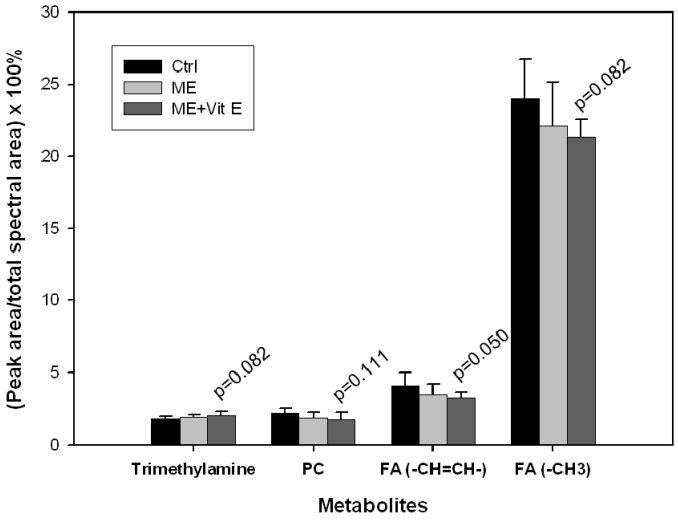

Fig. 6. Metabolic changes in the livers of rats exposed to motorcycle exhaust (ME) or ME + Vitamin (Vit) E compared with the control (Ctrl). Increased levels of trimethylamine and decreased levels of lipids were observed in the ME-treated and ME + Vit E-treated groups. PC (phosphatidylcholine) and FA (fatty acyl chain).

DISCUSSION

Effects of ME Treatment Alone and ME Plus Vit E Co-treatment on the Testes

ME contains volatile organic compounds, PAHs, as well as fine and ultrafine particles (Yang *et al.*, 2005a; Vasic and Weilenmann, 2006; Chang and Chen, 2008), which can travel deep into the lungs and cause systemic adverse effects. ME-mediated reproductive effects were first reported in our group using a rat inhalation study (Huang *et al.*, 2008). Although ME may not be the largest contributor to the very low birth rate in Taiwan, its effects on the health of the general population and subsequent generations are of public interest. In the present study, ME exposure caused decreases in testicular weight and spermatid number, which were partially attenuated by Vit E co-treatment. Vit E might protect sperm from oxidative stress or apoptosis during certain stages of spermatogenesis. The metabolic responses to ME or ME + Vit E treatment in the rat testes provided valuable information regarding the possible toxic mechanisms of action of ME. In our results, numerous amino acids, such as alanine, glycine, leucine, tyrosine, and valine, were consistently elevated in the testes of rats in the ME-exposed group. Betaine, ethanolamine, and inositol were significantly altered ($p < 0.05$) in the testes. Thus, our discussion mainly focuses on the roles of these metabolites and their potential to induce male reproductive toxicity.

Based on the results of the testicular weight and spermatid number analyses, ME treatment with or without Vit E generated adverse effects in the testes which were partially attenuated by Vit E co-treatment. These results were consistent with the testicular amino acid findings. Numerous amino acids, such as alanine, glycine, leucine, tyrosine, and valine, were elevated in the ME-treated group. These increases may have resulted from protein degradation or shifts in the energy supply when the testes were subjected to ME-induced stress. Elevated amino acid levels may provide an extra energy supply that allows organisms to maintain basic biological functions when they are under stress. The results of previous studies have demonstrated that alanine, leucine and valine are important substrates for Sertoli cell energy metabolism (Kaiser *et al.*, 2005). Although the full picture of the energetic supply in the process of spermatogenesis remains to be determined, amino acids are believed to play an important role. The increased amounts of amino acids were thought to have been contributed by either Sertoli cell or germ cells. Increases in amino acids have been reported in earthworms and salmon smolts exposed to PAHs (Jones *et al.*, 2008; Lin *et al.*, 2009a). After Vit E co-treatment, the changes in these testicular amino acids were potentially attenuated, indicating the anti-oxidative protective effects of Vit E. This protective mechanism ensures testicular function and the ability to adapt to additional stresses.

Our results demonstrated that the testicular betaine levels were significantly elevated in ME-treated rats, whereas the levels in the ME + Vit E-treated group were between those in the control and ME-treated groups. The results were negatively correlated with the sperm count trend among 3 groups. Betaine is an oxidized metabolite of choline, which is the substrate for the most abundant membrane lipids, phosphatidylcholine (PC) and sphingomyelin. High levels of unsaturated fatty acids on the sperm membrane provide ideal sites for peroxidative attacks, and, subsequently, spermatozoa lose membrane integrity and the ability to maintain sperm function and motility. In the present study, the appearance of lipid hydroperoxide in the NMR spectra of the testicular extracts was not related to any of the treatments. However, previous work has shown that ME causes a trend toward increasing lipid peroxidation, based on the results of a thiobarbituric acid assay in rat testes using the same exposure protocol (Huang *et al.*, 2008). Increased betaine levels in the ME-treated group may indicate that enhanced lipid catabolism occurred, thereby removing damaged lipids or disrupting lipid synthesis. With Vit E co-treatment, the effects of ME on betaine were partially reversed, indicating that Vit E may maintain lipid pool homeostasis.

The levels of two lipid synthesis substrates, inositol and ethanolamine, were found to have decreased in the testes of rats exposed to ME + Vit E co-treatment. Although the reduced levels of both metabolites were not statistically significant in the ME group, a similar trend was found in the ME group. Inositol is primarily synthesized in the testes via the circulation of glucose-6-phosphate by inositol-1-phosphate synthase, followed by inositol-1-phosphatase (Eisenberg, 1967). The levels of inositol increase as sperm travel from the testes to the epididymis, leading to the belief that inositol may play a role in spermatozoa maturation as they migrate from the testes (Eisenberg and Bolden, 1964). Moreover, the inositol concentration in the rete testis fluids of rams was also found to be positively correlated with spermatogenic activity (Setchell *et al.*, 1971). In the present study, the testicular inositol level was found to have decreased in rats that had been exposed to ME, with or without Vit E co-treatment. ME-induced adverse effects on spermatozoa maturation were suspected. Decreases in testicular inositol may lead to a lack of substrates for the biosynthesis of lipids, specifically phosphatidylinositol, that are required for sperm growth. Ethanolamine is a substrate for phosphatidylethanolamine (PE), which is a major phospholipid component and the second most abundant phospholipid in mammalian cellular membranes. The decreased ethanolamine levels in the testes may also suggest decreased PE biosynthesis, which could limit sperm growth.

The trend of reduced inositol and ethanolamine levels may partially explain the decreases in spermatid number in the rats that were exposed to ME, with or without Vit E co-treatment. The decrease was only statistically significant in the ME + Vit E group. The lack of significance in the ME group may be due to the limited sample size. It is also possible that the decrease is due to Vit E treatment. To identify the cause definitively, future studies should include a group that receives Vit E treatment alone. Moreover, the limited ability of Vit E to reverse the ME-mediated metabolic effects and effects on testicular sperm count suggests that mechanisms in addition to oxidative damage may be involved in male reproductive toxicity.

Effects of ME Treatment Alone and ME Plus Vit E Co-treatment on the Liver

Although ME does not induce significant injury in the rat liver, it alters many metabolic enzymes, such as CYP 1A1, superoxide dismutase, and catalase (Ueng *et al.*, 1998; Ueng *et al.*, 2004b), as well as increases susceptibility to lipid peroxidation. In the present study, decreases in PC, unsaturated fatty acyl chains (-CH=CH-), and fatty acyl chain terminals ($-CH_3$) were found, along with increased trimethylamine and trimethylamine N-oxide in the livers of ME-treated rats, especially those that had undergone Vit E co-treatment. Although these changes were not statistically significant, the p values of these metabolites in the livers were close to $p = 0.05$. Due to the small sample sizes in this study, the trends of these changes may indicate that certain biological effects have occurred. Trimethylamine, a product of lipid catabolism, can be further metabolized to trimethylamine N-oxide. The decreased PC, unsaturated fatty acyl chains (-CH=CH-), and fatty acyl chain terminal ($-CH_3$) levels in the livers of ME-exposed rats, despite Vit E treatment, suggested possible alterations in the balance of lipid catabolism by shifting PC toward increased trimethylamine N-oxide production. The results suggested that the induction of lipid degradation removed excessive lipids. Similar decreases in lipids that were accompanied by increases in lipid catabolism products were also observed in the livers of rats treated with alpha-naphthylisothiocyanate and high doses of triadimefon (Waters *et al.*, 2001; Ekman *et al.*, 2006).

The alterations in these metabolites were more significant in the livers of the ME + Vit E-treated group than in the group treated with ME alone. Whether the accelerated lipid metabolism caused by Vit E resulted from cellular stress or rejuvenation was not determined in this study.

Limitations

Because metabolomic studies generate huge and complex datasets, analysis that can provide a summary or overview of the observations in a simple and straightforward manner is essential. Our study design used a pattern recognition method to identify potential biomarkers in the datasets and further used a traditional statistical method to confirm the significance of the changes in these molecules. The strengths of OPLS-DA are that it enhances group separation and identifies potential biomarkers. In many cases, the variables (metabolites) contributing to the clustering may not differ statistically when analyzed using ANOVA. Our ANOVA results revealed that changes in the levels of numerous metabolites were close but not statistically significant, which is likely due to the small sample sizes. In addition, due to limited sensitivity of the NMR instrument, several low abundant and unknown metabolites, which might be important, were not included in our analysis. Therefore, one of the limitations of this study is the fact that low-abundance molecules related to the mechanism underlying toxicity may not have been identified. Although a trend of decreased levels of ethanolamine and inositol was found in all ME treated rats, it was only significant in the ME + Vit E group. A Vit E only group should be included in future studies to confirm the influence of Vit E on metabolic effects. Several publications have shown that multiple factors may affect metabolic characterization, including sex, strain, diurnal variations (Plumb *et al.*, 2003; Hines *et al.*, 2007), age (Plumb *et al.*, 2005), intestinal microflora (Williams *et al.*, 2002), and animal caging and handling (Robertson *et al.*, 2002). Therefore, we made every attempt to avoid variations in the experimental procedures. In addition, we concluded that the metabolome is sensitive and that the use of a high-throughput and unbiased metabolomic approach can reveal unexpected findings.

CONCLUSIONS

The effects of motorcycles on air quality and public health have been largely underestimated and ignored. The aims of the present study were to identify the molecular events underlying ME-induced male reproductive toxicity using a high-throughput and unbiased metabolomic approach. The proposed mechanism, the alteration of lipid metabolism and energy-related metabolism in the testes of ME-exposed rats, provided a hypothesis that can be tested in further studies. The decreased spermatid numbers and increased levels of betaine and amino acids in the ME-treated group were partially attenuated by treatment with Vit E. In addition to oxidative damage, other mechanisms may be involved in male reproductive toxicity. The inhibition of steroidogenesis was suggested to be a possible mechanism underlying ME-induced male reproductive toxicity; however, the testosterone signals in the NMR spectra of the testicular extracts were too low to be identified. Further studies of the effects of ME on steroidogenesis in both the testes and blood using an MS-based approach will aid in the mechanistic understanding of ME exposure and may lead to the further development of potential biomarkers that can be used for diagnostic purposes. Our hope is that this study will draw public attention to the potential adverse effects of ME on the male reproductive system. Further toxicological studies to characterize ME-induced male reproductive toxicity are essential for risk assessment and for the development of safer practices.

ACKNOWLEDGMENTS

The present study was supported by the National Science Council (NSC98-2314-002-085-MY3 and 97-2314-B-002-016-MY2) and the National Health Research Institutes (NHRI-EX99-9915EC, NHRI-EX100-9915EC, and NHRI-EX101-9915EC) of Taiwan. The authors would like to thank Chien-Han Chen for conducting the exposure experiments and Susie Huang and Yee Soon Ling for their assistance with writing the manuscript. The NMR spectra were obtained at the High-Field Nuclear Magnetic Resonance Center (HF-NMRC), which is supported by the National Research Program for Genomic Medicine. The ProMetab software program was kindly provided by Dr. Mark R. Viant.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declare no potential conflicts of interest

with respect to the research, authorship, and/or publication of this article.

SUPPLEMENTARY MATERIALS

Supplementary data associated with this article can be found in the online version at http://www.aaqr.org.

REFERENCES

Alirezaei, M., Jelodar, G. and Ghayemi, Z. (2012). Antioxidant Defense of Betaine Against Oxidative Stress Induced by Ethanol in the Rat Testes. *Int. J. Pept. Res. Ther.* 18: 239–247.

Beckonert, O., Keun, H.C., Ebbels, T.M.D., Bundy, J., Holmes, E., Lindon, J.C. and Nicholson, J.K. (2007). Metabolic Profiling, Metabolomic and Metabonomic Procedures for NMR Spectroscopy of Urine, Plasma, Serum and Tissue Extracts. *Nat. Protoc.* 2: 2692–2703.

Bligh, E.G. and Dyer, W.J. (1959). A rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* 37: 911–917.

Casu, M., Anderson, G.J., Choi, G. and Gibbons, W.A. (1991). NMR Lipid Profiles of Cells, Tissues and Body Fluids. I— 1D and 2D Proton NMR of Lipids from rat Liver. *Magn. Reson. Chem.* 29: 594–602.

Chang, C.T. and Chen, B.Y. (2008). Toxicity Assessment of Volatile Organic Compounds and Polycyclic Aromatic Hydrocarbons in Motorcycle Exhaust. *J. Hazard. Mater.* 153: 1262–1269.

Eisenberg, F. Jr. and Bolden, A.H. (1964). Reproductive Tract as Site of Synthesis and Secretion of Inositol in the Male Rat. *Nature* 202: 599–600.

Eisenberg, F. Jr. (1967). D-myoinositol 1-phosphate as Product of Cyclization of Glucose 6-phosphate and Substrate for a Specific Phosphatase in Rat Testis. *J. Biol. Chem.* 242: 1375–1382.

Ekman, D., Keun, H., Eads, C., Furnish, C., Murrell, R., Rockett, J. and Dix, D. (2006). Metabolomic Evaluation of Rat Liver and Testis to Characterize the Toxicity of Triazole Fungicides. *Metabolomics* 2: 63–73.

Fan, T.W.M. (1996). Metabolite Profiling by One and Two Dimensional NMR Analysis of Complex Mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* 28: 161–219.

Hines, A., Yeung, W.H., Craft, J., Brown, M., Kennedy, J., Bignell, J., Stentiford, G.D. and Viant, M.R. (2007). Comparison of Histological, Genetic, Metabolomics, and Lipid-based Methods for Sex Determination in Marine Mussels. *Anal. Biochem.* 369: 175–186.

Huang, J.Y., Liao, J.W., Liu, Y.C., Lu, S.Y., Chou, C.P., Chan, W.H., Chen, S.U. and Ueng, T.H. (2008). Motorcycle Exhaust Induces Reproductive Toxicity and Testicular Interleukin-6 in Male Rats. *Toxicol. Sci.* 103: 137–148.

Jones, O.A.H., Spurgeon, D.J., Svendsen, C. and Griffin, J.L. (2008). A Metabolomics Based Approach to Assessing the Toxicity of the Polyaromatic Hydrocarbon Pyrene to the Earthworm Lumbricus Rubellus. *Chemosphere* 71: 601–609.

Kaiser, G.R.R.F., Monteiro, S.C., Gelain, D.P., Souza, L.F., Perry, M.L.S. and Bernard, E.A. (2005). Metabolism of Amino Acids by Cultured Rat Sertoli Cells. *Metabolism* 54: 515–521.

Kuo, M.L., Jee, S.H., Chou, M.H. and Ueng, T.H. (1998). Involvement of Oxidative Stress in Motorcycle Exhaust Particle-induced DNA Damage and Inhibition of Intercellular Communication. *Mutat. Res.* 413: 143–150.

Lee, C.C., Liao, J.W. and Kang, J.J. (2004). Motorcycle Exhaust Particles Induce Airway Inflammation and Airway Hyperresponsiveness in BALB/C Mice. *Toxicol. Sci.* 79: 326–334.

Lin, C.Y., Wu, H., Tjeerdema, R.S. and Viant, M.R. (2007). Evaluation of Metabolite Extraction strategies from Tissue Samples Using NMR Metabolomics. *Metabolomics* 3: 55–67.

Lin, C.Y., Anderson, B.S., Phillips, B.M., Peng, A.C., Clark, S., Voorhees, J., Wu, H.D.I., Martin, M.J., McCall, J., Todd, C.R., Hsieh, F., Crane, D., Viant, M.R., Sowby, M.L. and Tjeerdema, R.S. (2009a). Characterization of the Metabolic Actions of Crude versus Dispersed Oil in Salmon Smolts via NMR-based Metabolomics. *Aquat. Toxicol.* 95: 230–238.

Lin, C.Y., Hung, P.H., VandeVoort, C.A. and Miller, M.G. (2009b). 1H NMR to Investigate Metabolism and Energy Supply in Rhesus Macaque Sperm. *Reprod. Toxicol.* 28: 75–80.

Ludwig, C. and Viant, M.R. (2010). Two-dimensional J-resolved NMR Spectroscopy: Review of a Key Methodology in the Metabolomics Toolbox. *Phytochem. Anal.* 21: 22–32.

Ong, E.S., Chor, C.F., Zou, L. and Ong, C.N. (2009). A Multi-analytical Approach for Metabolomic Profiling of Zebrafish (Danio rerio) Livers. *Mol. Biosyst.* 5: 288–298.

Oostendorp, M., Engelke, U.F., Willemsen, M.A. and Wevers, R.A. (2006). Diagnosing Inborn Errors of Lipid Metabolism with Proton Nuclear Magnetic Resonance Spectroscopy. *Clin. Chem.* 52: 1395–1405.

Pajunen, T.I., Koskela, H., Hase, T. and Hopia, A. (2008). NMR Properties of Conjugated Linoleic Acid (CLA) Methyl Ester Hydroperoxides. *Chem. Phys. Lipids* 154: 105–114.

Plumb, R., Granger, J., Stumpf, C., Wilson, I.D., Evans, J.A. and Lenz, E.M. (2003). Metabonomic Analysis of Mouse Urine by Liquid-chromatography-time of Flight Mass Spectrometry (LC-TOFMS): Detection of Strain, Diurnal and Gender Differences. *Analyst* 128: 819–823.

Plumb, R.S., Granger, J.H., Stumpf, C.L., Johnson, K.A., Smith, B.W., Gaulitz, S., Wilson, I.D. and Castro-Perez, J. (2005). A Rapid Screening Approach to Metabonomics Using UPLC and oa-TOF Mass Spectrometry: Application to Age, Gender and Diurnal Variation in Normal/Zucker Obese Rats and Black, White and Nude Mice. *Analyst* 130: 844–849.

R.O.C. (2012). Number of Registered Motor Vehicles., Vol. 2013. Ministry of Transportation and Communications R.O.C.

Robertson, D.G., Reily, M., Lindon, J.C., Holmes, E. and Nicholson, J.K. (2002). Metabonomic Technology as a

Tool for Rapid Throughput in Vivo Toxicity Screening. *Compr. Toxicol.* 14: 583–610.

Santos, E.M., Ball, J.S., Williams, T.D., Wu, H., Ortega, F., van Aerle, R., Katsiadaki, I., Falciani, F., Viant, M.R., Chipman, J.K. and Tyler, C.R. (2009). Identifying Health Impacts of Exposure to Copper Using Transcriptomics and Metabolomics in a Fish Model. *Environ. Sci. Technol.* 44: 820–826.

Setchell, B.P., Voglmayr, J.K. and Hinks, N.T. (1971). The Effect of Local Heating on the Flow and Composition of Rete Testis Fluid in the Conscious Ram. *J. Reprod. Fertil.* 24: 81–89.

Trygg, J. and Wold, S. (2002). Orthogonal Projections to Latent Structures (O-PLS). *J. Chemom.* 16: 119–128.

Tugnoli, V., Bottura, G., Fini, G., Reggiani, A., Tinti, A., Trinchero, A. and Tosi, M.R. (2003). 1H-NMR and 13C-NMR Lipid Profiles of Human Renal Tissues. *Biopolymers* 72: 86–95.

Ueng, T.H., Hwang, W.P., Chen, R.M., Wang, H.W., Kuo, M.L., Park, S.S. and Guengerich, F.P. (1998). Effects of Motorcycle Exhaust on Cytochrome P-450-dependent Monooxygenases and Glutathione S-transferase in Rat Tissues. *J. Toxicol. Environ. Health A.* 54: 509–527.

Ueng, T.H., Wang, H.W., Huang, Y.P. and Hung, C.C. (2004a). Antiestrogenic Effects of Motorcycle Exhaust Particulate in MCF-7 Human Breast Cancer Cells and Immature Female Rats. *Arch. Environ. Contam. Toxicol.* 46: 454–462.

Ueng, T.H., Wang, H.W., Hung, C.C. and Chang, H.L. (2004b). Effects of Motorcycle Exhaust Inhalation Exposure on Cytochrome P-450 2B1, Antioxidant Enzymes, and Lipid Peroxidation in Rat Liver and Lung. *J. Toxicol. Environ. Health A.* 67: 875–888.

Ueng, T.H., Hung, C.C., Kuo, M.L., Chan, P.K., Hu, S.H., Yang, P.C. and Chang, L.W. (2005). Induction of Fibroblast Growth Factor-9 and Interleukin-1alpha Gene Expression by Motorcycle Exhaust Particulate Extracts And Benzo(A)Pyrene In human lung adenocarcinoma Cells. *Toxicol. Sci.* 87: 483–496.

Vasic, A.M. and Weilenmann, M. (2006). Comparison of Real-world Emissions from Two-wheelers and Passenger Cars. *Environ. Sci. Technol.* 40: 149–154.

Viant, M.R. (2003). Improved Methods for the Acquisition and Interpretation of NMR Metabolomic Data. *Biochem. Biophys. Res. Commun.* 310: 943–948.

Viant, M.R., Rosenblum, E.S. and Tieerdema, R.S. (2003). NMR-based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. *Environ. Sci. Technol.* 37: 4982–4989.

Waters, N.J., Holmes, E., Waterfield, C.J., Farrant, R.D. and Nicholson, J.K. (2002). NMR and Pattern Recognition Studies on Liver extracts and Intact Livers from Rats Treated with α-naphthylisothiocyanate. *Biochem. Pharmacol.* 64: 67–77.

Waters, N.J., Holmes, E., Williams, A., Waterfield, C.J., Farrant, R.D. and Nicholson, J.K. (2001). NMR and Pattern Recognition Studies on the Time-Related Metabolic Effects of α-Naphthylisothiocyanate on Liver, Urine, and Plasma in the Rat: An Integrative Metabonomic Approach. *Chem. Res. Toxicol.* 14: 1401–1412.

Williams, R.E., Eyton-Jones, H.W., Farnworth, M.J., Gallagher, R. and Provan, W.M. (2002). Effect of Intestinal Microflora on the Urinary Metabolic Profile of Rats: A (1)H-nuclear Magnetic Resonance Spectroscopy Study. *Xenobiotica* 32: 783–794.

Yang, H.H., Chien, S.M., Chao, M.R. and Lin, C.C. (2005a). Particle size Distribution of Polycyclic Aromatic Hydrocarbons in Motorcycle Exhaust Emissions. *J. Hazard. Mater.* 125: 154–159.

Yang, H.H., Hsieh, L.T., Liu, H.C. and Mi, H.H. (2005b). Polycyclic Aromatic Hydrocarbon Emissions from Motorcycles. *Atmos. Environ.* 39: 17–25.

Zhou, W. and Ye, S.H. (1997). Mutagenicity of Scooter Exhaust Particulate Matter. *J. Toxicol. Environ. Health* 52: 35–44.

Received for review, September 5, 2013
Accepted, February 14, 2014