Regular Article

Biomarker Identification of Maternal Genistein Exposure Induced Obesity by Metabonomics Analysis

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The objective of this study was to confirm the effect of maternal genistein exposure on body weight of male offspring and the metabolic alterations associated with maternal genistein-induced obesity. Pregnant female Sprague–Dawley (SD) rats were supplemented with 300 mg/kg diet of genistein (GEN) or no genistein (CON) throughout pregnancy and lactation. The growth of male offspring was investigated until 12 week age and the mechanism of obesity was studied using metabonomics by ultra performance liquid chromatography and quadrupole time-of-flight (UPLC Q-TOF) MS with electrospray ionization in positive ESI mode (ESI+). Compared with the CON group, body weight, fat pad and food intake of male offspring in GEN group were increased significantly at the age of weeks 10 to 12 (p<0.05). Ten urine principal metabolites contributing to the clusters were identified, including increased 8-Isoprostaglandin F2a, and decreased l-Proline, Betaine, l-Acetylcarnitine, Norsalsolinol, Indoleacrylic acid, l-Tryptophan, Lysophosphatidylcholines (LysoPC) (20 : 4), Lysophosphatidylethanolamines (LysoPE) (18 : 1) and LysoPC (O-18 : 0). Our results confirmed weight-increasing effects of maternal genistein exposure, accompanied by favorable changes in metabolites in the male offspring urine. Therefore, this research enables us to better understand obesity and predict risk of obesity-related disease by studying metabolites present in the urine.

Key words genistein; body weight; maternal exposure; metabolic profile; obesity; lysophosphatidylcholine (LysoPC)

With the development of the social economy, both in developed and developing countries, obesity is becoming an increasingly concerned epidemic worldwide disease. With the increased prevalence of obesity, other chronic non-communicable diseases associated with obesity triggered an obvious increase in the trend, such as diabetes, metabolic syndrome, hypertension, cardiovascular disease, sleep disorders, asthma as well as cancer.1)

Genistein is a natural isoflavone compound found in leguminous plants and dentate plants. It is an A-trihydroxy compound with aromatic a-rings (Fig. S1). Previous studies have revealed the optimal extraction technology for genistein.2,3) Genistein is known to have multiple molecular effects, such as the inhibition of inflammation, promotion of apoptosis, and modulation of steroidal hormone receptors and metabolic pathways. Our team found genistein affected obesity in different animal models. For example: genistein could influence glucose metabolism on high fat diet-induced insulin resistance and diabetes in ovariectomized rats.4) Furthermore, genistein could reduce body weight by decreasing food intake in ovariectomized rats by regulating hypothalamus and peripheral orexigenic gene expression.5,6) Nowadays, we focus on the early-exposure of genistein on obesity of offspring and its mechanism. Some research revealed that maternal genistein could decrease body weight while others suggested a higher body weight when consumed in pregnancy and lactation.7–9) Previous results of our team showed that a high dose of genistein exposure to dams could reduce body weight in male pups while no change in female offspring. However, the mechanism is not clear.

Recently, investigators have been using metabonomics to profile serum or urine in search of biomarkers and to explore the mechanisms of intraperitoneal chemicals exposure and diseases, such as maternal vitamin D supplement and children's vitamin D deficiency,10) intrauterine lipopolysaccharide exposure and brain injuries in offspring.11) Additionally, a number of methods are currently used to assess the effect of maternal genistein exposure to their offspring, including epidemiological surveys2,12) and different animal experiments.13–16) However, none of these tested the changes of metabolism.

In the current study, we carried out a urinary metabonomics study on male offspring to identify reliable biomarkers and to explore the metabolic alterations associated with the maternal genistein exposure.

MATERIALS AND METHODS

Chemicals Genistein was purchased from Xi'an Rongsheng Bio-Technology Co., Ltd. (Xi'an, China) with purity=99.45%. Each group of rats was fed with this diet with or without genistein (GEN: 300 mg genistein/kg diet; CON: 0 mg genistein/kg diet).

Procedure Prior to study initiation, the experimental protocol was reviewed and approved by the Committee on Animal Research and Ethics of Harbin Medical University (Harbin, China). Twenty-four female Sprague-Dawley (SD) rats (10 weeks, 220–250 g) and 12 male rats (10 weeks, 250–280 g) were purchased from Beijing WTLH Laboratory Animal Co., Ltd. of China (Batch No: SCXK (jing) 2012-0001). The animals were caged in a room maintained at 21±2°C with a 12-h light/dark schedule. After adaptation for 1 week, the rats were randomly assigned to 2 groups prior to mating. Pregnancy was determined by the presence of vaginal sperm plug. Female rats were exposed daily during pregnancy and lactation with preparations of genistein or control in diets. Pups whose mother delivered * To whom correspondence should be addressed. e-mail: naxiaolin1495@sohu.com; chengwang1359@163.com © 2018 The Pharmaceutical Society of Japan
10–15 rats were reserved. Each mother was preserved 6 rats for enough breast milk. After weaning, 16 pups (8 males/8 females) were selected randomly and received a diet without genistein treated until 12 weeks. Body weight was measured weekly. At the end of the experiment, urine of every rat was collected, centrifuged with 3000rpm for 15 min and stored at −80°C. Rats were anesthetized and sacrificed, parametrical and perirenal fat pads of rats were dissected and weighed to calculate the ratio of fat pad weight. The formula was as follows: ratio of fat pad weight (the ratio of fat pad weight×100/body weight).

**Blood Lipids** Blood was collected from the ventral aorta after anesthetized with chloral hydrate (350mg/kg body weight). Then blood was transferred to 15mL tubes immediately and centrifuged at 4°C (3000 rpm, 15 min). A 2µL serum was used for every blood lipids detection such as total cholesterol TC, Triglycerides TG, High-density lipoproteins HDL, Very-low-density lipoproteins VLDL and Low-density lipoproteins LDL with Automatic Analyzer (Hitachi7100, Japan). The reagent kits were bought from Maccura biological company (Sichuan, China).

**Urine Sample Preparation** Urine samples were allowed to thaw at 4°C, diluted 1:1 (v/v) with purified water prepared with electrical resistance of 18.2Ω (Millipore, U.S.A.), mixed by vortex for 1 min, and then separated by centrifugation at 12000rpm (13362 g) for 10 min. The supernatants were then transferred into autosampler vials.

**Ultra-Performance Liquid Chromatography (UPLC)**

Analysis UPLC-MS analysis was performed with a Waters Acquity UPLC system coupled to a Waters Micromass Q-TOF micro™ mass spectrometer with electrospray ionization (ESI) in the positive modes. The detailed parameters were followed with previous reports.17

In addition, we used urine samples (quality control, QC) to assess reproducibility and reliability of the UPLC-MS system. The QC samples were prepared by mixing equal volumes of different individual urine samples (8/GEN group and 8/CON group). One QC sample was injected at the start of the analytical batch, followed by analysis of 1 QC sample at every fourth sample injection throughout the analytical workflow. The reproducibility of the method was determined with principal component analysis (PCA) (Fig. S2), and reliability was assessed by extracting 4 ions from chromatographic peaks of the QC samples (Table S1).

**Data Analysis** Statistical analysis was performed using SPSS (version 13.1S; Beijing Stats Data Mining Co., Ltd., Beijing, China). Data was presented as mean±standard deviation (S.D.). Differences between groups were analyzed using the independent samples t-test. All p-values were 2-tailed and a p-value <0.05 was considered to be significant.

Metabolite identification based on MS/MS analysis was followed as previous research17 with 864 peaks. Sequentially, the implicated pathways of biomarkers were interpreted using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca), including Human Metabolome Database (HMDB) (http://www.hmdb.ca) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/).

**RESULTS**

**Body Weight, Fat Pad Weight and Food Intake** The effect of maternal genistein exposure on the changes of male offspring body weight, ratio of fat pad weight and food intake was shown in Fig. 1. Compared with the CON group, body weight of male offspring in GEN group was not different from postnatal week 0 to week 9. However, from postnatal week 10, body weight of GEN group increased significantly compared with CON group (p<0.05) until week 12 (Fig. 1A). Meanwhile, food intake in male offspring was increased markedly in GEN group from week 9 to week 12 (p<0.05) compared with the CON group (Fig. 1C). In addition, the ratio of fat pad weight/body weight of GEN group increased significantly compared with the CON group (p<0.05) (Fig. 1B).

**Blood Lipids** Blood lipids such as TC, TG, HDL, LDL and VLDL were detected to investigate the effect of maternal genistein exposure on lipid metabolism of male offspring. However, the blood lipids did not change obviously (data not shown).

**UPLC Quadrupole Time-of-Flight (Q-TOF) MS Fingerprinting and Multivariate Analysis** The partial least squares discriminant analysis (PLS-DA) score plot showed a separation along the axes corresponding to the 2 PLS-DA components (Fig. 2A). The variation in X (R²X) was 47.5%, while the variation in Y (R²Y) was 94.7% which predicts 78.0% of the variation in response to Y (Q²Y=78.0%) for 2-component model. The permutation test with a permutation number of 200 was performed and indicated a R² intercept value of 0.406 and a Q² intercept value of −0.338 (Fig. 2B).

**Potential Biomarkers** According to the selection standards, we identified 10 metabolites (Table 1). The concentrations of specific metabolites were significantly higher in GEN

![Fig. 1. Effect of Maternal Genistein Exposure on Body Weight (A), Fat Pad Weight (B) and Food Intake (C) of Male Offspring
GEN: 300mg genistein/kg diet, CON: control group (0mg genistein/kg diet). *p-value <0.05 was considered significantly compared with the CON group.](image-url)
rats compared with the CON group, including 8-Isoprostaglandin F2a, whereas other metabolites, such as L-Proline, Betaine, L-acetylcarnitin, Norsalsolinol, Indoleacrylic acid, L-Tryptophan, Lyso phosphatidylycholines (LysoPC) (20:4), Lyso phosphatidylethanolamines (LysoPE) (18:1) and LysoPC (O-18:0) were significantly lower in GEN group. Mass fragment information of principal metabolites with different collision energy (ev) was shown in Fig. S3.

Pathway Analysis  When the impact of metabolic pathways’ affect >0.1, the pathway was recognized as significance. In this study, pathway involved was ether lipid metabolism and tryptophan metabolism (Fig. 3).

DISCUSSION
In this study, the results showed that maternal genistein exposure could increase body weight, fat pad weight and food intake of male offspring at their adult age. However, the
blood lipids were not altered. This is to say, maternal genistein exposure could promote the development of obesity without significant blood lipids changes. Previous study revealed that 50mg/kg maternal genistein exposure (from postnatal day 1 to postnatal day 22) favored the development of obesity in female, but not male rats in their adult age (postnatal day 97), while another work found that 3600mg genistein maternal exposure could reduce body weight in male pups at their adult age (week 8). The main differences between these studies were the differences of dosage and administration routes. It indicates that maternal genistein exposure has bidirectional regulation on body weight, that is early-life exposure to a high dose of genistein could reduce body weight while a low dose of genistein could increase body weight at their adult age.

Metabonomics analysis revealed 10 metabolites and 2 significant pathways were included to represent the possible mechanism for body weight change. Ether lipid metabolism is related to phosphoglycerides metabolism normally. Obese individuals come along with dyslipidemia usually. However, in present study, no significant blood lipids were changed in obese rats, which suggested other phosphoglycerides metabolism might be changed. Lyso phosphatidylcholines (lysoPC) and lyso phosphatidylethanolamines (lysoPE) are phospholipids which are important component of biofilm participating in various biological pathways. Notably, Our results suggested that 3 metabolites were lower in obese rats with maternal genistein exposure, mainly lysoPC and lysoPE, such as LysoPC (18:1), LysoPC (20:4), and LysoPC (O-18:0).

Tryptophan (TRP) metabolism controlled serotonin availability, mood alterations, T-cell mediated inflammation and clinical endpoints of Cardiovascular disease (CVD). Pro-inflammatory cytokines activated the enzyme indoleamine 2,3-dioxygenase (IDO), which decreased serum levels of the essential amino acid tryptophan. Obesity is known an inflammatory process and often has increased serum kynurenine to tryptophan ratios, a result of an increased TRP breakdown. In present study, TRP and Indoleacrylic acid level were reduced in obese rats with maternal genistein exposure, which suggested inflammatory process was involved in the process of obesity. Furthermore, tryptophan breakdown leads to mood, appetite and sleep dysregulation, which may be the reason of increased food intake in obese rats with maternal genistein exposure.

Amino acid metabolism provides material basis for protein synthesis, energy metabolism and other maintenance of normal metabolism. Proline, the only proteinogenic secondary amino acid, regulates key metabolic pathways that are necessary for maintenance, growth, reproduction, and immunity. Research showed abnormal urinary proline was associated with metabolic disease such as iminoglycinuria and hyperglycinuria. Betaine is an important member of glycine metabolism pathway. Reports showed that betaine insufficiency was associated with metabolic syndrome, lipid disorders, and diabetes, and may have a role in vascular and other disease. In present study, the levels of l-proline and betaine in maternal genistein exposure group were lower than control group, which indicated metabolic changes might be involved in the appearance of obesity induced by maternal genistein exposure. Norsalsolinol is a chemical compound that is produced naturally in the body through metabolism of dopamine. Research found obese individuals had lower level of dopamine, which lead to more consumption of food in obese people to get more pleasure. Our results showed an increase consumption of food in obese male offspring induced by maternal genistein exposure.

8-Iso-prostaglandin F2a is the best studied F2-isoprostane which represents the most reliable and accurate markers of oxidative stress status in vivo. Elevated levels of urinary 8-iso-prostaglandin F2a have been reported in inflammatory, metabolic and degenerative diseases including type 2 diabetes, insulin resistance, cardiovascular disease and coronary heart diseases. 1-acylcarntine, is an acetic acid ester of carnitine that facilitates movement of acetyl-CoA into the matrices of mammalian mitochondria during the oxidation of fatty acids. Reports showed abnormal metabolites of 1-acylcarntine was associated with obesity, colorectal adenoma, crohns disease and ulcerative colitis. Although no significant change was investigated in blood lipids, the elevation of 8-iso-prostaglandin F2a and decreased 1-acylcarntine in male rats with maternal genistein exposure may be an indicator of above diseases.

To the best of our knowledge, this is the first report to evaluate the metabolic changes in male rats after maternal genistein exposure using UPLC/Q-TOF MS. In the present study, some other principal metabolites were detected but remain unidentified at present. There were some limitations to our studies. We used only male rats in the animal study, thus, the gender may have been a confounder in the metabonomics experiments. Moreover, the changes in animals are not the same as human. Large, well-designed studies that are needed to verify in humans the potential application of these biomarkers from our animal models.

CONCLUSION

In summary, we have confirmed that maternal genistein
exposure could increase body weight and food consumption in their male offspring. In addition, we have identified potential biomarkers of obesity induced by maternal genistein exposure for the first time. These metabolites changes are indicative of early changes in metabolic disorders, insulin resistance, type 2 diabetes and cardiovascular disease. However, whether these changes are suitable for human beings is still needed to be further studied.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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