Metabonomic Analysis of *Bombyx mori* (Heterocera: Bombysiidae) Treated With Acetaminophen

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**ABSTRACT.** The feasibility of using *Bombyx mori* as model animal is attracting more attention. Whether the effect of drugs on the metabolite profiling was consistent with those in mammals was an aspect to evaluate the feasibility of *B. mori* as model animal. In this study, we used acetaminophen to treat Dazao fifth-instar *B. mori*, and its metabolites in hemolymph were detected by gas chromatography—mass spectrometry. The corresponding data were processed and analyzed by total model analysis, principal component analysis, partial least squares-discriminant analysis, orthogonal partial least squares-discriminant analysis, and finally, the difference metabolites between acetaminophen group and control group were selected and identified by our reference material database and the National Institute of Standard and Technology database. The results showed that acetaminophen administration induced elevation of metabolites related to energy source, the intermediate of cholesterol synthesis, and the metabolites related to melanization and also induced the decrease of metabolites in pathway of Krebs cycle, the cholecalciferol, and sitosterol, which suggested that acetaminophen administration inhibited energy metabolism and promoted the expenditure and imbalance of hormone and melanization.

**Key Words:** acetaminophen, *Bombyx mori*, horme synthesis, Krebs cycle, metabolite
chromatography–mass spectrometry (GC/MS), and analyzed data by metabolonomic analysis. The results showed that acetaminophen administration induced elevation of metabolites related to energy source, the intermediate of cholesterol synthesis, and the metabolites related to melanization and also induced the decrease of metabolites in pathway of Krebs cycle, the cholesterol, and sitosterol, which suggested that acetaminophen administration promoted the energy metabolism, the expenditure, and imbalance of hormone and melanization.

Materials and Methods

**B. mori Feeding and Hemolymph Obtainment.** *B. mori* were fed mulberry leaves for 12:12 (L:D) h at 25°C each day. Fifth-instar *B. mori* were divided into two groups (n = 10): acetaminophen-treated group (AP group) and control check group (CK group). Two groups of *B. mori* were fasted for 4 h, and acetaminophen-treated group received acetaminophen by intragastric injection administration. Then two groups were fed as common. Ten hours later, 10 hemolymph samples from each group were used in GC/MS detection, and 7 quality-control samples were treated at the same time.

**Sample Pretreatment.** Twenty microliters of hemolymph sample were added to 80 μl of ice-cold methanol, vortex for 30 s, kept at −20°C for 20 min, and centrifuged for 15 min at 14,000 *g*, 4°C. Eighty microliters of supernatant were added into glass vial and blowed dry by nitrogen to obtain dry matter. Dry matter was added into thirty microliters of 20 mg/ml methoxamine hydrochloride pyridine solution, oximation reaction for 90 min, then added 30 μl of O-Bis (trimethylsilyl) acetamide N (containing 1% trimethylchlorosilane) derivatizing reagent, kept closed at 70°C for 20 min, and centrifuged for 15 min at 14,000 *g*, 4°C. Finally, sample was taken out and kept at room temperature for 30 min for GC/MS.

**GC/MS Analysis.** Agilent 7890A/5975C GC/MS system and HP-5 capillary chromatogram column (Agilent J&W Scientific, Palo Alto, CA) (30 m by 0.25 mm by 0.5 μm) were used in detection. The parameters were as follows: injector temperature: 250°C; EI ionization source temperature: 230°C; quadrupole temperature: 150°C; high purity helium (purity >99.999%) as carrier gas; and sample volume: 1.0 μl. Temperature program: beginning temperature 80°C for 2 min, 10°C/min up to 320°C for 6 min. Full-scan model was used in mass detection (range 50–600 m/z). Random sequence was used in continued sample analysis to avoid the disturbance of signal fluctuation.

**Data Process.** Raw data were pretreated by our program including baseline filtering, peak identification, integration, retention time correction, peak alignment, and mass fragment classification and then edited in excel. The final data were two-dimension data matrix including variable (rt, mz, i.e., retention time, mass to charge ratio), sample, and integration area. Five hundred thirty-four matters were obtained. All data were normalized to total signal integration. All edited data matrix was imported into Simca-P software 11.0 for PCA, PLS-DA, and OPLS-DA.

**Analysis Setting.** Data were processed by Unit Variance Scaling and mean centered to obtain straightforward results in Simca-P software. Data were analyzed by automodel fitting to obtain reliable number of preliminary component. Also, cross-validation (n = 7) was used to establish model for preventing model from overfitting.

**Screening and Identification of Difference Metabolites.** Difference metabolites were obtained by combining variable importance in the projection of the first preliminary component in OPLS-DA model with *P* value (threshold 0.05) in Student’s *t*-test. The identification of difference metabolites was done by searching own reference chemical data and the National Institute of Standard and Technology commercial data (comparing retention time or retention index of mass spectrum and chromatograph).

**Results and Discussions**

**Review of Chromatogram.** All total ion chromatograms were reviewed, and the results showed strong signal, large peak capacity, and good reproducibility for retention time. The representative total ion chromatogram is shown in Fig. 1.

**General Model Analysis.** The principal component analysis for samples shows the reliability of test method in general, the metabolic difference among groups, and the viability of samples within group. This study firstly did PCA for CK group sample, AP group sample, and quality control (QC) sample and obtained five principal components; the cumulative rate of interpretation is 0.682. Generally, the cumulative rate of interpretation is >0.4 means the reliability of model, cumulative rate of interpretation 0.682 shows this model is reliable. Hence, the model established by us could be used to test the metabolic difference among groups in general. PCA scores plot is shown in Fig. 2A. In Fig. 2A, t[1] (x-axis) and t[2] (y-axis) represent the scores plot of the first principal component and the scores plot of the second principal component, respectively. The scores plot showed QC sample gathered in a narrow principal component plot (*), while CK and AP samples scattered in a wide principal component plot (△, ■). CK samples scattered in a wider plot than AP samples, which indicated that variation in CK was higher than AP group, thus suggested that the influence on *B. mori* mainly resulted from drug (acetaminophen) treatment. In further analysis, QC samples were excluded.

**PCA Between AP and CK Group.** Four principal components were obtained from fitting analysis by Simca-P software, and the cumulative rate of interpretation is 0.602, which suggested the reliability of model. The scores plot of PCA is shown in Fig. 2B, and all samples were within 95% confidence interval (Hotelling T² ellipse) and showed no outlier samples. Figure 2B showed PCA scores plot of AP and CK group located lower left side and upper right side, respectively, which suggested that two groups had significant metabolic difference.

**PLS-DA Between AP Group and CK Group.** Two principal component by PLS-DA method, *R*²Y = 0.987, *Q*² = 0.943, the scores plot is shown in Fig. 2C. *t*[1] (x-axis) and *t*[2] (y-axis) represented the scores plot of the first principal component and the scores plot of the second principal component, respectively. *R*²Y is the interpretation rate of model and is 0.987, which suggested that PLS-DA model could interpret the difference between two groups appropriately. Also, the predictive rate of model (*Q*²) was 0.943, which showed good predictive capacity. Figure 2C showed that samples from two groups were located in different sites, which suggested that two samples from two groups had significant metabolic difference.

**OPLS-DA Between AP Group and CK Group.** One principal component (P, *R*²Y = 0.888) and one orthogonal component (O, *R*²Y = 0.099) were calculated from OPLS-DA analysis, and its quality index was *R*²Y = 0.987, *Q*² = 0.91, which showed that OPLS-DA model was very reliable. The scores plot is shown in Fig. 2D. Two samples from AP group and CK group were located in the plus side and minus side, respectively, which indicated that two groups had significant metabolic difference in OPLS-DA scores plot.

**Difference Metabolites and Their Structure Identification Between AP Group and CK Group.** Difference metabolites were very reliable because of filtering irrelevant orthosignal. Difference metabolites were obtained by combining variable importance in the projection of the first principal component in OPLS-DA model (threshold > 1) with *P* value (threshold 0.05) in Student’s *t*-test. In this study, 41 difference metabolites were obtained by screening, the level of 18 metabolites decreased and 23 metabolites increased.

We screened the metabolites with the absolute value of fold change > 0.5 and obtained 10 metabolites. We found these 10 metabolites were mainly related to three metabolic processes: glycolysis and Krebs cycle, steroid, and melanization. Then we selected metabolites related to these three processes and obtained 14 metabolites that showed the metabolic change induced by acetaminophen in *B. mori* was mainly related to carbohydrate catabolism, hormone metabolism, and pigmentation (Table 1). The levels of substances involved in Krebs cycle including fumaric acid, α-ketoglutaric acid, malic acid, and succinic acid were decreased significantly, and the corresponding fold change [log₂(AP/CK)] was −0.49, −0.25, −0.47, and −0.34, respectively. The level of trihalose, glucose, and β-alanine increased, and fold
change $\log_2(\text{AP}/\text{CK})$ was 2.17, 2.03, and 1.27, respectively. The level of the intermediate for cholesterol, beta-hydroxy-beta-methylglutaric acid, increased (fold change $\log_2(\text{AP}/\text{CK})$ 0.67), while fold change $\log_2(\text{AP}/\text{CK})$ of cholesterol and sitosterol was $-0.86$ and $-0.51$, respectively. The levels of the metabolites related to melanization, tyrosine and dopa, increased, and their fold changes were $\infty$ and 2.59, respectively.

**Discussions**

To explore the effect of the acetaminophen on the metabolite profile in *B. mori*, we detected the metabolites in hemolymph of *B. mori* treated with acetaminophen (AP group) or with normal saline (CK group) using GC/MS, then found the significant difference of metabolites between AP group and CK group by PCA, PLS-DA, and OPLS-DA. Forty-one difference metabolites between AP group and CK group were identified using databases. These difference metabolites were mainly related to Krebs cycle, hormone, and melanization.

Glycine is derived from serine, which is derived from 3-phospho-D-glycerate, an intermediate of glycolysis. Tryptophan can be oxidized into the precursor of nicotinamide adenine dinucleotide (NAD) and NAD phosphate (NADP). When tryptophan changes into precursor of NAD and NADP, the catabolism of glycogen decreases. In our study, the level of glycine and tryptophan decreased, which suggested the depletion of substances in glycolysis and the inhibition in glycolysis.

The levels of substances involved in Krebs cycle including fumaric acid, $\alpha$-ketoglutaric acid, malic acid, and succinic acid were decreased significantly, which suggested that acetaminophen administration might inhibit catabolism. Besides, the level of glucose, trehalose, and $\beta$-alanine increased, which indicated that acetaminophen administration induced higher glucose, trehalose, and $\beta$-alanine. Trehalose is a main blood sugar for insect, which is synthesized by fat body of *B. mori* and hydrolyzed into two molecule of glucose to be absorbed and used by organism. Beta-alanine is the intermediate product in Krebs cycle and can transfer into pyruvic acid and enter into Krebs cycle. Therefore, the changes of above substances generally suggested that acetaminophen administration inhibited energy consuming and producing. In vitro experiment showed that acetaminophen inhibited NADH-linked respiration reversely, whereas the metabolites inhibited all mitochondrial respiration, apparently in the complex III region of the respiratory in mouse liver mitochondria (Ramsay et al. 1998). In vivo study for CD-1 mice showed that inhibition of mitochondrial respiration, especially glutamate- and succinate-supported respiration, was an early event in acetaminophen-induced hepatotoxicity (Donnelly et al. 1994). Also, the metabonomic analysis of urine and plasma in chimeric mice and normal mice revealed alterations of endogenous metabolites, which were the intermediates involved in the Krebs cycle (Yamamoto et al. 2007). Our study also showed the similar results as the above study, which suggested that the influence of the acetaminophen on Krebs cycle might be similar or conserved in *B. mori* and mammals.

Xiao et al. (2009) reported exogenous trehalose was positive in increasing the resistibility of silkworm and suggested that trehalose might be an important carbohydrate involved in stress metabolism. In our study, acetaminophen induced higher level of trehalose, which also suggested that trehalose might play a role in stress metabolism.

Cholesterol widely exists in animal and is necessary for multiple physiological activities. Beta-hydroxy-beta-methylglutaric acid is the
intermediate for cholesterol. Gu et al. (2013) reported that the cholesterol level of fifth-instar *B. mori* was lower than other phases. In our study, acetaminophen induced lower cholesterol level and higher beta-hydroxy-beta-methylglutaric acid, which suggested that acetaminophen administration induced cholesterol expenditure for more stress physiological activities. Sitosterol belongs to lipids, and it is the precursor of ecydson, which is not synthesized by *B. mori*, must be uptaken from food. Beta-sitosterol is the sterol preference of *B. mori* in a few sterols including beta-sitosterol, ergosterol, cholesterol, and stigmasterol (Nagata et al. 2006). Acetaminophen induced lower level of sitosterol, and suggested acetaminophen administration led to imbalance of sterol in *B. mori*.

Tyrosine hydroxylase can catalyze L-tyrosine into L-dopa, which is the precursor for dopamine and melanin. Liu et al. (2010) reported that repression of tyrosine hydroxylase is responsible for the sex-linked chocolate mutation of the silkworm, *B. mori*. In our study, acetaminophen administration induced higher level of tyrosine and dopa, which suggested that acetaminophen promoted pigmentation of *B. mori*.

In summary, we used acetaminophen to treat *B. mori*, and analyzed the metabolic profile of hemolymph, the results showed that there were difference metabolites between group treated with acetaminophen and

### Table 1. The influence of acetaminophen administration on the metabolics of *B. Mori*

| Compounds                     | VIP value (OPLS-DA) | P value (t-test) | Fold change*<sub>log₂(AP/CK)</sub> |
|-------------------------------|---------------------|-----------------|-------------------------------------|
| Glycolysis and Krebs cycle    |                     |                 |                                     |
| Glycine                       | 1.75                | 1.08E-04        | -0.99                               |
| Tryptophan                    | 1.52                | 1.59E-03        | -0.73                               |
| Fumaric acid                  | 1.71                | 1.75E-04        | -0.49                               |
| a-Ketoglutaric acid           | 1.48                | 2.25E-03        | -0.25                               |
| Malic acid                    | 2.00                | 6.67E-07        | -0.47                               |
| Succinic acid                 | 1.68                | 2.59E-04        | -0.34                               |
| Glucose                       | 2.01                | 7.89E-08        | 2.03                                |
| Trehalose                     | 1.49                | 4.74E-05        | 2.17                                |
| β-Alanine                     | 1.74                | 1.15E-04        | 1.27                                |
| Sterol                        |                     |                 |                                     |
| Cholesterol                   | 1.26                | 1.25E-02        | 0.86                                |
| Sitosterol                    | 1.04                | 4.51E-02        | -0.51                               |
| β-Hydroxy-β-methylglutaric acid | 1.64             | 4.16E-04        | 0.67                                |
| Melanization                  |                     |                 |                                     |
| Tyrosine                      | 1.35                |                 | ∞                                   |
| Dopa                          | 1.56                | 1.03E-03        | 2.59                                |

VIP, variable importance in the projection.

*The binary logarithm for the ratio of AP to CK.

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**Fig. 2.** Scores plot of PCA for CK, AP, and QC groups (A) and scores plots of PCA (B), PLS-DA (C), and OPLS-DA(D) for CK and AP groups. t[1] represents the first principal component, t[2] represents the second principal component, t[1]P represents principal component of OPLS-DA, and t[2]O represents orthogonal component of OPLS-DA.
control group, and the difference metabolites included substances in glucose glycolysis and Krebs cycle, metabolite-related sterol and melanization. This study is a preliminary study for B. mori as replacement animal, and the detailed metabolites induced by acetaminophen between B. mori and mice need to be studied.

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