Silver effects on silkworm, *Bombyx mori*

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(Received June 1, 2018; Accepted August 27, 2018)

**ABSTRACT** — Silver nanoparticles (Ag-NPs) are known as a noble metal, and owing to their exclusive properties, their use is widespread in consumer products and they are mostly incorporated into food packaging and food contact products. The aim of this work was to evaluate the effects of direct ingestion of Ag-NPs through food to assess their toxicity effects on the growth and development of silkworms at different concentrations (1 mg·L⁻¹ to 100 mg·L⁻¹), in addition to the examination of the distribution of Ag-NPs in the silkworm body and midgut histopathological analysis. RNA sequencing was performed to investigate the transcriptomic responses to Ag-NPs exposure. Our results show that the highest Ag-NPs' concentrations induced a significant increase in the silkworm body weight with histopathological changes in the midgut compared to the control group. The gene ontology (GO) and pathway enrichment analysis for differentially expressed genes showed that Ag-NPs altered the gene expressions and that they were significantly involved in carbohydrate metabolism, digestive system, and energy metabolism. These findings indicate that the Ag-NPs may induce harmful effects on the primary target organs (alimentary system) with energy deregulation and nutrition digestion and absorption imbalance. This study is an important step in understanding the molecular mechanisms of Ag-NPs' toxicity in vivo.

**Key words:** Silkworm (*Bombyx mori*), Silver nanoparticles, Nanotoxicity

**INTRODUCTION**

Silver nanoparticles are among the nanoparticles that have been forcefully imposed themselves in nanotechnology field because of their antimicrobial activity. Ag-NPs are widely used in different areas including food packing (plastic bags, containers, films, and pallets) (Duncan, 2011; Carew et al., 2015), medical applications, for instance, wound dressings (Faunce and Watal, 2010), and stents, breathing tubes, cardiovascular implants, drug delivery, and dental instruments (Schlesener and Schlesener, 2013; Ge et al., 2014), besides other uses in detergents, cosmetics (Bondarenko et al., 2013), to name a few. However, despite the silver NPs’ advantages, many studies either in vivo or in vitro demonstrated their potential toxicity, and a wide range of model organisms, including bacteria, algae (Ivask et al., 2014) and Caenorhabditis elegans (Ellegaard-Jensen et al., 2012), have been used to evaluate their toxicity impact. Results indicated that exposure to silver nanoparticles may induce organs and tissues damage (Kim and Ryu, 2013). Recent studies showed that Ag-NPs may induce oxidative stress through ROS generation, DNA damage, apoptosis (Hadrup et al., 2012), and neurotoxic effects (Kaito and Sekimizu, 2007).

The silkworm, *Bombyx mori* (*B. mori*), is known to be one of the important insects. It has been domesticated over 5000 years in China. Besides their advantages in the industrial field, recently, it is considered as an ideal insect model in various research fields, such as bacterial pathogenicity, the therapeutic effects of antibiotics and diabetic pharmacokinetics test (Sekimizu et al., 2012; Zhang et al., 2012). Furthermore, *Bombyx mori* has been employed for evaluation of drug toxicology effects, and studies have proved that it is very sensitive to toxic substances (Meyer et al., 2010).

In this study, we attempt to establish a set of methodologies for nanoparticles toxicity detection and safety testing on silkworms. The breakthrough point of this study is to set nanosilver as a research object to evaluate the nanotoxicity in vivo through feeding (oral administration). And the aim of this study is to establish a rapid and low-cost technological system using *Bombyx mori* to evaluate potential hazards of nanosilver and to investigate their impact, fate, and underlying mechanisms of toxicity.
MATERIALS AND METHODS

Method for suspending Ag-NPs
Silver nanoparticles powder was purchased from Suzhou Nord Derivatives Pharm-Tech Co. Ltd. (Suzhou, China). Ag-NPs stock solution was synthesized as previously described (Yu et al., 2015). The Ag-NPs powder was dispersed in deionized water at 100 mg·L⁻¹ and the mixture was treated with ultrasonication at 100 W and 40 kHz for 25 min to prepare the stock solution, which was diluted with deionized water to the selected dose range (1 mg·L⁻¹ to 100 mg·L⁻¹). The concentrations were chosen after determining survival to the adult stage.

The characterization and morphology of prepared Ag-NPs were examined by transmission electron microscopy (TEM, JEM-2100, JEOL, Tokyo, Japan) and are summarized in Fig. 1.

Experimental exposure method
Silkworms, *Bombyx mori*, domestic strain, Qing-Song × Hao-Yue, were cultured under short-day conditions (12 hr light/12 hr dark) in the rearing chamber. Larvae were fed on fresh mulberry leaves in the growth chamber, which was maintained at a temperature of 25°C, from the beginning till exposure time.

From the first day of fifth instar, worms were exposed to silver nanoparticles until mounting, by using the leaf dipping method. Briefly, from the first day of fifth instar, worms were fed with mulberry leaves that were soaked in different concentrations of Ag-NPs and dried naturally at room temperature (Zhang et al., 2014). Five concentrations (1, 10, 25, 50, and 100 mg·L⁻¹) were set up and we used mulberry leaves treated with pure water as a control. The control and exposure groups were fed three times a day. The test was replicated three times and each handling contained 30 silkworms.

The development and histopathological assessment of silkworm digestive systems
During the exposure time to silver NPs, the body weight of *B. mori* larvae was recorded each 24 hr, for control or exposure worms, in which the mass of *B. mori* was measured with an analytical balance. In addition, the movement, and mortality of silkworm after the exposure

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**Table 1.**

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**Fig. 1.** Physiochemical characterization of silver nanoparticles. (a) Summary of some properties of silver nanoparticles. (b) TEM images of Ag-NPs. (c) Energy-dispersive X-ray spectroscopy (EDS). (d) The particle-size distribution (PSD) of Ag-NPs dispersed in deionized water.
to silver nanoparticles were observed and recorded.

In order to examine the impact of silver NPs on silkworm tissues, the samples were collected at the end of the last treatment (96 hr), at which the fifth instar larval were randomly selected from each group, and the histopathological evaluation of midgut was employed. Briefly, the worms were anesthetized and then dissected in Insect Ringer’s solution (28 mM NaCl, 1.8 mM CaCl₂, 1.3 mM KCl, and 0.05 M Tris). The midguts of the control and treated groups were embedded in paraffin blocks, then sliced (5 µm thickness), and placed on glass slides. After hematoxylin-eosin staining, the stained sections were evaluated by a histopathologist who was unaware of the treatments, using an optical microscope (Leica DMi6000 B, Wetzlar, Germany).

Distribution and translocation assessment of silver nanomaterials in silkworm body

Silkworms’ fifth instar was reared up to the first day on normal mulberry leaves. Then we modified the feed containing Rhodamine B (Sigma-Aldrich, St. Louis, MO, USA), labeled the silver nanomaterial basically as previously described, in short Rho B was loaded on the Ag-NPs by mixing Rho B solution (1 mg·mL⁻¹, 0.3 mL) with an aqueous suspension of Ag-NPs (0.1 mg·mL⁻¹, 5 mL) basically as previously described (Nouara et al., 2013). Unbound Rho B was removed by dialysis against distilled water over 72 hr. The resulting AgNPs/Rho was stored at 4°C. The examined worms were fed the modified mulberry leaves. This treatment was applied at different terms, 16 hr, 36 hr, and 48 hr until they started spinning their cocoons, to examine the possibility of the translocation of nanomaterials in the hemolymph, after being absorbed from the gastrointestinal tract. The extraction of hemolymph from the control and treated groups was examined. Briefly, after the incubation time, 10 instar larvae were selected randomly and chilled in crushed ice for 10 min. The hemolymph was harvested from the legs, and it was collected into sterile vials containing phenol thiourea, which prevents melanization of the hemolymph (Han et al., 2015). The hemolymph cells were observed with a laser scanning confocal microscope (Leica TCS SP5 II, Wetzlar, Germany). The characteristic fluorescence of Rho B was detected at the emission wavelengths of λex 553nm, λem 627 nm. Meanwhile, we observed the change in the color of the silkworm’s body observed under room light conditions, in addition to the dissection of the silkworm midgut and silk gland carried out in pH 6.2 Insect Ringer’s solution to detect the translocation of the nanosilver dye to organs.

RNA sequencing analysis

To analyze the transcriptome profile of the silkworm midgut, after 96 hr of feeding with Ag-NPs, the midgut was dissected from two silkworm groups (100 mg·L⁻¹ and control) and frozen in liquid nitrogen for storage and stored at –80°C. Library construction and deep sequencing were performed by Genenergy Biotechnology Co. Ltd. (Shanghai, China) using Illumina HiSeq 2000, following the manufacturer’s instructions (Illumina).

There exists a list of all genes/transcripts as a background, with gene/transcription of this list being as filtered out from the background list of candidate lists, calculated using the hypergeometric distribution test. Gene/transcript on behalf of GO feature set is a significant enrichment of p values in the list, and then on the p value obtained by Benjamini and Hochberg after multiple testing correction FDR (FDR < 0.05 was used as the threshold to determine significant enrichment of the gene sets). Hypergeometric distribution test formulas to calculate p values are as follows:

\[ P = 1 - \sum_{i=0}^{m-1} \binom{N-m}{n-i} \binom{m}{i} \]

Where \( N \) represents gene/transcript in GO annotations of gene number; \( n \) represents the number of DGEs in N; \( M \) represents gene/transcript annotations in a GO gene/transcript number; and \( m \) represents differences in gene/transcript annotations in a GO gene/transcript number.

The gene expression profiles between the treated silkworms (100 mg·L⁻¹) and the control were performed to investigate the differentially expressed genes (DEGs), GO categories, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The fragments per kilobase of exon model per million mapped reads (FPKM) were calculated to investigate gene expression abundance using the Cuffnorm program, which is used to calculate the sample expression Log2. The threshold for the genes that showed significant enrichment in the fixed list of genes in this analysis (the gene sets was 2-fold and FDR ≤ 0.05 cutoff for differential expression identification p ≤ 0.05).

GO enrichment analysis of DEGs was implemented using functional annotation, and GO analysis was carried out using the free GO classification system (http://www.geneontology.org/). According to a p value less than or equal to 0.05, screening significantly enriched GO and displayed up to 10 GO. The abscissa represents -log10 (p value), while the ordinate represents a significant enrichment of GO under the name of each category.

To have a better understanding of the biological responses to nanosilver, the KEGG database (http://www.genome.jp/kegg) was used to detect the functional anno-
tations of the identified genes, in which the pathways with $P \leq 0.05$ were considered significantly enriched pathways.

**Statistical analysis**

The statistical analysis was conducted by using SPSS 11.7 and the method of analysis of variant (one-way ANOVA) followed by Dunnett’s t-test for comparisons between groups. $P$-value $< 0.05$ was considered significant compared to the control. All data were expressed as mean $\pm$ standard error of the mean (SEM). The reproducibility of all examined bioassays was confirmed at least three times with different populations of larvae.

**RESULTS**

**Characterization of Ag-NPs**

The structure of Ag-NPs in deionized water after sonication was analyzed by transmission electron microscopy (TEM) (Fig. 1b). The results showed spherical features of Ag-NPs with an average size of 20 nm. Data on Ag-NPs indicated the presence of Cu, MO, and Fe impurities at concentrations of not more than 0.001% and 0.003% for Ni as determined by energy-dispersive X-ray spectroscopy (EDS) (Fig. 1c), more information in Supplementary file.

**The body weight and survival rate**

The toxic effects of silver NPs on the growth and development of silkworms were investigated for five different concentrations as follows: 1, 10, 25, 50, and 100 mg·L$^{-1}$. The silkworms showed a slight change in color compared to the control group (Figs. 3a-b). With increases in the silver concentration, we observed an increase in the body weight of silkworm larva during the exposure time. The exposure to the highest concentration (100 mg·L$^{-1}$) showed a very significant increase ($p < 0.05$) in the body weight compared to the control sample after 96 hr (Fig. 2; Fig. 3c). However, the *B. mori* larvae did not show any significant toxicity symptoms at all concentrations (1-100 mg·L$^{-1}$) during the exposure time, in either movement or reproduction (data not shown). Furthermore, Ag-NPs did not affect the survival rate of larvae even at 100 mg·L$^{-1}$, the highest exposure concentration, as shown in Fig. S1 (Supplementary file), suggesting that Ag-NPs may induce morphological alteration but could not cause

![Fig. 2. Effects of silver NPs on silkworm body weight in the body of fifth instar larvae of *B. mori*. Weight of silkworm larva after exposure to silver NPs at different concentrations compared to control group. Asterisks (*) indicate significant differences ($p < 0.05$) in the body weight compared to the control sample at 100 mg·L$^{-1}$.

![Fig. 3. Comparison of body pigmentation and body size between nonexposed silkworms and exposed silkworms (the fifth instar larvae) after exposure to silver NPs (100 mg·L$^{-1}$) at 96 hr, (a) treated *B. mori*, (b) control, (c) body size of control and silkworms exposed to Ag-NPs (100 mg·L$^{-1}$).](image-url)
mortality to silkworm larvae.

**Transverse section of midgut tissue**

The histopathological evaluation of midgut samples after 96 hr of treatment with different concentrations ranging from 1 to 100 mg L⁻¹ showed that the Ag-NPs induced particular changes in the microscopic structure compared to the control sample. As shown in Fig. 4, the treated groups displayed significant abnormal pathology, especially at 10, 25, 50, and 100 mg L⁻¹, including damage in the basal lamina, death cell-like columnar cells, goblet cells, and vacuolization with karyopyknosis and decrease in nucleus. This result indicated that Ag-NPs caused adverse effects on tissues and may potentially induce harmful effects on the primary target organs (alimentary system) of *Bombyx mori*.

**Distribution and translocation assessment of silver nanomaterials in silkworm body**

In order to investigate the distribution and translocation of silver nanoparticles in the silkworm body, from the third day of their fifth instar we fed them the silver nanoparticles labeled Rhodamine B (Ag-NPs-Rho B). We monitored the fate of Ag-NPs-Rho B by the observation of the change in color of silkworm’s body as well as the silk gland and gut after the dissection at different time points under room light. At the first 16 hr, we observed that the color changed slightly in the body and organs, as displayed in Fig. 5 (I), and, progressively, we noticed that the pink color increased in the silkworm’s body, which became significantly pink at 48 hr compared to control with normal feed as shown in Figs. 5 (II), a, b, c. Meanwhile, the intensity of color gradually increased in the gland silk and gut as displayed in Fig. 5 (II), a. To verify this observation more, the Ag-NPs-Rho B was tracked in the hemolymph by using confocal laser scanning microscopy (CLSM). The observation indicated that the nanosilver crossed the digestive membrane barrier and translocated to the circulatory system hemolymph and entered the hemocytes (Fig. 6). Notably, the fluorescence intensity of Rho B decreased with time, which may be due to the migration of silver NPs to other tissues or because they were excreted through the excretory system. This result indicated that the ingestion of Ag-NPs can not only be retained in the digestive tract, but also be translocated to the hemolymph, reaching other second target organs and tissues, such as the silk gland, the fat body, and Malpighian tubule. However, we did not observe any change either in the color of the cocoon or in the adult moth as displayed in Figs. 5 (II), b.

**Bioinformatics analysis**

RNA sequencing is a new technology and has many advantages with high sensitivity to understand clear-
Fig. 5. The uptake and distribution of Rhodamine B-Ag-NPs in the body of fifth instar larvae of *B. mori* after 48 hr of exposure. (I) the distribution of Rhodamine B-Ag-NPs in organs at different time points (0 hr, 16 hr, 36 hr, 48 hr) under room light conditions. (II); the (a) a pink silkworms for Rhodamine B-Ag-NPs after 48 hr of exposure, (c) silkworm with normal diet, (b) the cocoon and adult moth of fifth instar larvae fed Rhodamine B-Ag-NPs from second day until molting.

Fig. 6. Translocation and distribution of Rhodamine B-Ag-NPs in the hemolymph cells of the fifth instar larvae of *B. mori* at different time points (16 hr, 36 hr, 48 hr), which compared with a control group the nanosilver crossed the primary target barrier and migrated to the circulatory system (hemolymph) and entered the hemocytes (granulocytes, plasmocytes).
ly the global transcriptome profile, including differential gene expression, alternative splicing, pathways and SNP, and so forth (Bellmann et al., 2015). We found herein, by using RNA sequencing and computational approaches, that silver NPs altered gene expression in the silkworm midgut after 96 hr of treatment at 100 mg·L⁻¹. The analysis showed that there were 251 significant (p < 0.05) DEGs, 172 upregulated and 79 downregulated. The genes that are highly significant with p < 1.0E-04 were further classified as shown in Table S1 (Supplementary file). To gain insight into the molecular mechanisms of silver NPs toxicity, the GO and KEGG analyses of the DEGs were performed. We conducted the GO enrichment analysis, using all the GO terms listed in Supplementary Table 2 (Supplementary file).

The result of GO enrichment analysis revealed that there were 668 terms enriched, among which 82 were significant GO annotations (p < 0.05), including 41 terms in biological process ontology, 37 terms in molecular function ontology, and 4 terms in cellular component ontology. The GO terms and gene descriptions are shown in Table S2 (Supplementary file).

The GO analysis found that these DEGs between control and nanosilver groups were significantly enriched in transmembrane transport, carbohydrate metabolic process, single-organism transport alcohol catabolic process, organic hydroxy compound catabolic process, galactose metabolic process, localization, carbohydrate phosphorylation, cellular carbohydrate metabolic process, carbohydrate kinase activity, transporter activity, hydrolase activity, hydrolyzing O-glycosyl compounds, and hydrolase activity, acting on glycosyl bonds and transmembrane transporter activity as illustrated in Fig. 7.

The KEGG analysis results demonstrated that nanosilver affected several pathways (107 terms in total). A full list of all regulated processes is provided in Supplementary Table 3, among which the top 30 ranking pathways of most variable KEGG categories are illustrated in Fig. 8. The significant (p < 0.05) deregulations were observed in galactose metabolism, nitrogen metabolism, amino sugar and nucleotide sugar metabolism, selenocompound metabolism, sulfur metabolism, carbohydrate digestion and absorption microbial metabolism in diverse environments, butirosin and neomycin biosynthesis, complement
and coagulation cascades, pancreatic secretion, neuroactive ligand-receptor interaction, D-glutamine and D-glutamate metabolism, ECM-receptor interaction, alanine, and aspartate and glutamate metabolism, in addition to GABAergic synapse (p > 0.05).

**DISCUSSION**

**Toxic effects of silver NPs on growth and development of silkworm**

Previous research indicated that the digestive tract can be the major route of nanomaterials entry into a body (Aillon et al., 2009). However, after nanomaterials enter the body, there are numerous factors that may interfere with their consequences (Blaut, 2015). Early studies pointed out that the gut microbiota plays a very important role in metabolic processes to maintain healthy food track, such as their effective function in the energy balance (Rai et al., 2012). Thus, the interaction between the nanoparticles and microbiota is very important and may cause metabolic diseases in the host. Silver NPs is one of the nanoparticles that may be ingested because it may relate to food products and dietary supplements as well as medical products. According to previous studies, silver nanoparticles have great antimicrobial properties, for example, on fungus, bacteria, and virus (Hadrup et al., 2012), which can alter the membrane function of microorganism and block the electron transport, leading to a further significant impact on DNA replication (Williams et al., 2015).

The present study clearly demonstrated the harmful impacts of silver NPs on the silkworm. Our results showed an obese phenotype and a significant increase in silkworm weight after treatment with nanosilver at different concentrations ranging from 1 to 100 mg·L⁻¹ (Fig. 2). We suggest that the antimicrobial effects of silver NPs might affect the microbiota of silkworm gut and that is why it was probably causing imbalance in the bacterial diversity, resulting in alterations in the production of digestive enzymes as well as in the other metabolic activities, such as the nutrition digestion and absorption, the energy regulation, and the immune system function. However, we did not report any effect of Ag-NPs on the life span of silkworm at all concentrations that have been used. In a recent study, Williams K. and his colleagues revealed that exposure to silver NPs induced an alteration in intestinal microbiota of Sprague-Dawley rats, such as reductions in Firmicutes phylum, *Lactobacillus* genus population, as well as dysregulation of gene expression, especially the genes involved in immune responses (Elkloub et al., 2015). Kout Elkloub et al. found that broilers fed on a diet supplemented with silver nanoparticles showed weight gains, particularly by the addition of 4 ppm of Ag-NPs/kg. No adverse effects on the microflora were found. Meanwhile, a decrease was observed in harmful bacteria, namely, *Escherichia coli*. Therefore, the author suggested that nanosilver is supplementing at 4 ppm Ag-NPs/kg in broiler diets (Subramanian et al., 2009).

So far, many studies have presented silkworm (*B. mori*) gut microbiota as having a considerable diversity, particularly with the development of genomic techniques (PCR probes based on 16S rRNA genes) that helped discover new strains of bacteria (Liang et al., 2014). It has been reported that there are several factors that may influence the variability of the gut microbiota in the silkworm, including the developmental phase of worms, the diet, such as the age of the mulberry leaves (Sekar et al., 2010; Sun et al., 2016), and region of silkworm gut (Sun et al., 2016). Some of the bacterial strains that have been isolated from silkworm midgut were Gram-positive *Bacillus circulans* and Gram-negative *Escherichia coli* and *Pseudomonas fluorescens* (Sun et al., 2016; Modi et al., 2014). Interestingly, our results regarding the Ag-NPs effects on silkworm morphology (body weight) and our hypothesis that the ingestion of nanosilver contributed to the development of obesity in silkworm regarding the antibacterial impact of Ag-NPs on bacterial communities in the gut were in accordance with previous reports. Several studies indicated that the antibiotics may change the gut microbiota (Bailey et al., 2014) in a negative manner and, accordingly, cause gain weight (Cermenati et al., 2007).

**The histopathological evaluation of midgut samples**

The midgut represents the primary targeted organs and the main site of digestion and absorption of materials in silkworm *B. mori* larvae (Franzetti et al., 2015). Several studies indicated the protective function of midgut against foreign and toxic compounds, through the release of enzymes (Zhang et al., 2014; Li et al., 2017; Wang et al., 2015). However, the digestion of toxic substances that may interfere with the composition of their diet unintentionally, such as the pesticide organophosphorus (OP), induced a serious adverse effect on midgut tissues (Shahare and Yashpal, 2013), including karyopyknosis and disappearance of the basal lamina, cell apoptosis (Zhang et al., 2014). Our observation was consistent with previous studies that showed that Ag-NPs may induce hazardous effects on the midgut tissues after 96 hr of treatment at different concentrations (1 mg·L⁻¹ to 100 mg·L⁻¹). The histological observation showed that the
Silver effects on silkworms

midgut of worms exposed to silver nanoparticles exhibited significant histopathological changes compared to the control group. As shown in Fig. 4, the treated groups showed significant disorganization of the normal appearance of testicular architecture and abnormal pathological, especially at 100 mg·L⁻¹. In a different study, analysis of Ag-NPs effects in mice showed that treatment with Ag-NPs induced significant changes in the mucosa of the small intestines; namely, it caused microvilli cells membrane damage as well as epithelial cells damage, in addition to inflammation in lamina propria cells. It was suggested that Ag-NPs may cross the small intestine barrier and induce disabled small intestine function (Oliveira et al., 2013). Ag-NPs also harmed the histological structure of lung and brain in goldfish (Chavan et al., 2015). The present study’s results revealed that the silver NPs may induce adverse effects on gut tissue and may harm the alimentary system of the silkworm, Bombyx mori.

Distribution and translocation assessment of silver nanomaterials in silkworm body

The midgut is considered as the primary target organ in silkworm, in which the midgut wall is composed of an inner epithelial layer and outer muscle layer, and the epithelium is differentiated into three cells (Nakahara et al., 2010). We chose to test if nanosilver will translocate to hemolymph, with regard to the fact that silkworm has an open circulatory system, and test further the effective role of hemocytes in immune response through phagocytosis of foreigner substances and toxic compounds. Silkworm, B. mori, hemolymph, is contained in different hemocytes, mainly granulocytes, plasmacytes, and oenocytoids (Tang et al., 2009). The observation under a laser confocal fluorescence microscope proved the ability of silver NPs to pass from the digestive tract to the hemolymph and penetrate to the hemocytes. In this study, at least two morphologically different cell types were detected in the hemolymph of silkworm, including granulocytes and plasmacytes. Notably, these cells showed phagocytic activity, with a significant encapsulation of silver NPs beads in granulocytes during the first 16 hr after ingestion, and at 36 hr; this observation obviously demonstrated the aggregation and infiltration of Ag-NPs to the nucleus (Fig. 6). However, the phagocytosis of foreign bodies disappeared imperceptibly after 48 hr. We suggest that the silver NPs might be excreted by the excretory system or might translocate to other tissues, after time.

However, this hypothesis needs more confirmation to know the fate of silver nanoparticles in the silkworm’s body after they reach the hemolymph. And we can conclude that the hemocytes (granulocytes and plasmacytes) identified the silver NPs as foreign molecules. Thus, silver NPs induced immune responses through the phagocytosis activity.

This result is in line with previous studies. Tang et al. (2009) reported that, after subcutaneous injection of silver nanoparticles in rats, the silver trafficked to the blood circulation and numerous organs, including the kidney, liver, spleen, brain, and lung, where silver NPs could enter cells, such as renal tubular epithelial cells and hepatocytic cells (Hendrickson et al., 2016). The results of several studies proved the ability of Ag-NPs to translocate and distribute into a body through the penetration of the primary membrane of the gastrointestinal tract to the circulatory system and the other organs (Jin et al., 2013). However, the underlying mechanism of translocation of silver nanoparticles remains not so clear. Furthermore, the discoloration of silkworm body, silk gland, and gut by the pink color under room lighting conditions indicates the translocation and spread of Ag-NPs-Rho.B throughout the silkworm’s body (Fig. 5 (II), a). Our results demonstrated that the Ag-NPs can induce adverse effects through reaching different organs in a short time. However, the pink color has not been observed either in the cocoons or in the moths under room lighting conditions, demonstrating the possibility that the Ag-NPs may not translocate to the second generation. However, the point of Ag-NPs effects on the next generation still needs more examination to assist in the fate of silver NPs and the translocation mechanism of Ag-NPs.

GO categories, KEGG pathway categories, and DEG in silkworm midgut

In the present study, the RNA sequencing analysis demonstrated that exposure to silver NPs affected significantly the gene expression in the silkworm midgut, and based on gene ontology, most of the genes belong to the biological processes, molecular function, and cellular component, respectively, in which most of these genes were upregulated in response to silver NPs treatment. The results of GO enrichment indicated that multiple biological processes were involved in the response to Ag-NPs exposure filling the metabolism function. We found that silver NPs affect significantly the carbohydrate metabolism and the membrane transport mechanisms in the silkworm midgut. Carbohydrate metabolism is the chemical process to break down carbohydrates into energy; therefore, it is an important pathway for cellular energy, in which any abnormalities in carbohydrate metabolism may lead to an imbalance in health issues (Wang et al., 2011). In contrast, only 4 terms were enriched for
the cellular component. These results suggest that silver NPs affected significantly the metabolic functions in the silkworm. Pathway analysis revealed 14 significantly altered pathways (Fig. 8). The majority of these altered pathways were related to metabolism categories, such as galactose metabolism, amino sugar, and nucleotide sugar metabolism, and energy metabolism as nitrogen metabolism and sulfur metabolism, and digestive system as carbohydrate digestion and absorption and pancreatic secretion, in addition to the immune system pathway category, as the complement and coagulation cascades. The galactose metabolism has been often one of the pathways that was significantly enriched with \( p < 2.2 \times 10^{-5} \), and so on. Among the differently expressed genes, genes related to carbohydrate metabolism were all upregulated, such as the GLCNACASE3 gene. In contrast, the genes related to the digestive system and energy metabolism were mostly upregulated, except BGIBMGA006555 and BGIBMGA014491, which were downregulated.

In addition, among the 251 differentially expressed genes, there were 232 genes found with unknown functions and 19 genes with known functions (Table 1). Some of them encode putative cuticle proteins, such as CPH37, CPH38, CPH40, CPH41, and CPG36. The cuticle is considered a protective cover for insects and can be involved in metabolic activities, too. In the present study’s results, the expression of four putative cuticle proteins was increased in response to silver NPs exposure. The explanation for this observation is that these genes were upregulated in response to silver nanoparticles effects and injury and they may be involved in the recovery process of those damaged on the cuticle. Further, they may be involved in the cuticular pigmentation alteration. However, CPG36 showed a decrease in expression in this study. Furthermore, we observed the upregulation of genes involved in the 30K family of proteins. Previous studies demonstrated that 30K-family proteins were found in the silkworm, synthesized specifically by the fat body, and that they play a very important role in energy metabolism. Interestingly, this family of proteins may be involved in apoptosis inhibition as has been reported (Park et al., 2003; Manjunatha et al., 2010). Therefore, we suggest that the upregulation of these genes was responding to apoptosis effects of silver NPs on the silkworm.

The heat shock protein family is composed of several subfamilies, including HSP20. HSP20 is a small heat shock protein. This protein may be involved in several cellular functions (Manjunatha et al., 2010). In this study, the silkworm, *Bombyx mori*, increased the synthesis of stress proteins HSP20.4. The HSP20.4 gene was
upregulated to about 2-fold, to respond to the stress that may be induced because of the alteration by feeding of silver NPs. Furthermore, this gene was involved in protein processing in endoplasmic reticulum pathway (p value not significant; Table S3 (Supplementary file). Glutathione peroxidase (GPx) is involved in the antioxidant defense in silkworm and is considered as an antioxidant enzyme biomarker (Muthusamy and Rajakumar, 2016). The present study’s results indicate that GPx was increased to overcome the toxic effects of silver. It has been reported that the glutathione peroxidase can play an important role in protecting cells from the reactive oxygen species induced by a pesticide (Muthusamy and Rajakumar, 2016).

Previous studies demonstrated that neuropeptide F (NPF) in insects, a vertebrate neuropeptide Y (NPY) homolog, plays a key role in the feeding behavior, stress responses, and homeostasis regulation (Slade and Staveley, 2016; Nässel and Wegener, 2011). The distribution of NPF has been first detected in the Drosophila brain as well as in the midgut (Slade and Staveley, 2016). Therefore, it was attributed that this gene plays a role in the feeding control (Nässel and Wegener, 2011), in which the neuropeptide F (NPF) deregulation may lead to eating disorders. The overexpression of NPF led to an increase in the food intake; conversely, the lack of NPF I signaling negatively affected the appetite (Wu et al., 2003). Notably, the expression of NPF is dependent upon the larva age, in which a higher NPF expression was found in younger larvae and vice versa (Wu et al., 2003). The silkworm has two genes, npf 1 and npf 2, which are located on chromosomes 6 and 2, respectively (Roller et al., 2008). The transcripts of neuropeptide F were detected in the brain and midgut endocrine cells of Bombyx mori (Roller et al., 2008). In this study, the exposure to silver NPs led to the upregulation of NPF2 (2.6-fold change), and we suggest a link between the overexpression of neuropeptide F (NPF) and the overweight of silk worm at 100 mg·L⁻¹. And it is most likely that the silver NPs affected the crosstalk between the brain and gut, including the gut microbiota of the silkworm, as we have been previously suggested. In contrast, the present KEGG pathways analysis identified that NPF2 was involved in adipocytokine signaling pathway (P > 0.05). The dysregulation of adipocytokine signaling pathway was implicated in insulin resistance, obesity, and inflammation (Zhao et al., 2013). However, the role of neuropeptide F remains unclear in silkworm feeding behavior and still needs more investigation. And it is noteworthy to mention that the trypsin-like serine protease (BMO.6705) showed overexpression more than 6-fold. Trypsin proteinases play a functional role during the digestion of food in insects (Sui et al., 2009) suggesting that it is very likely that the overexpression of trypsin-like serine protease after food inges-

### Table 1. Identification of 19 differentially regulated genes with known functions in the control and treatment (100 mg/L Ag-NPs) group.

<table>
<thead>
<tr>
<th>gene</th>
<th>gene_id</th>
<th>locus</th>
<th>p_value</th>
<th>Express</th>
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</table>

a Gene expression: “↑”upregulated expression; “↓” downregulated expression
tion was because the silver NPs may affect the behavior food intake in silkworms, leading them to increase the digestive enzymes as noted above, due to the alteration of the diversity in the microbiota gut to meet the digestive enzyme and process needs.

In summary, the GO enrichment terms and the pathways based on the enriched genes were highly consistent with the silkworms' morphological change. Our results suggest that it is very probable that the overexpression of the genes such as those involved in stress response and feeding behavior was due to the toxic stress effects of silver NPs to meet the energy required and to regulate the alteration in the function and metabolism that were induced by feeding of silver nanoparticles. However, more functional works need to explain this observation and to determine whether silver NPs are involved in the mechanisms underlying feeding behavior of silkworm and whether their antimicrobial properties affected the microbiota gut of the silkworms. One of the conclusions made from these results was that the alteration of the gene expression was to maintain the homeostasis and balance of internal environment in the silkworm’s body. Furthermore, the present study provides insight into the interaction between the host genes and food components. However, these results still need more assessment with more emphasis on molecular mechanisms of Ag-NPs toxicity. Based on these results, we propose the silkworm as a new model organism in future assessment of nanotoxicity.

ACKNOWLEDGMENT

This work was supported by the Project of the Priority Academic Program Development of Jiangsu Higher Education Institutions, the National Natural Science Foundation of China (No. 31572467, No. 31272507, 31570150, 31602008 and 31550110210), the National Natural Science Foundation of Jiangsu Province (BK20150495), Postdoctoral Science Foundation of Jiangsu Province (1501012B), and Postgraduate Research and Innovation Project of Jiangsu Province (No. CXLX12-0671).

Conflict of interest——The authors declare that there is no conflict of interest.

REFERENCES


Silver effects on silkworms


