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Titanium Dioxide Nanoparticles Relieve Silk Gland Damage and Increase Cocooning of *Bombyx mori* under Phoxim-Induced Toxicity

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ABSTRACT: Organophosphate pesticides are applied widely in the world for agricultural purposes, and their exposures often resulted in non-cocooning of *Bombyx mori* in China. TiO_2 nanoparticles have been demonstrated to increase pesticide resistance of *Bombyx mori*. While the toxicity of phoxim is well-documented, very limited information exists on the mechanisms of TiO_2 nanoparticles improving the cocooning function of *Bombyx mori* following exposure to phoxim. The present study was, therefore, undertaken to determine whether TiO_2 nanoparticles attenuate silk gland injury and elevate cocooning of *B. mori* following exposure to phoxim. The findings suggested that phoxim exposure resulted in severe damages of the silk gland structure and significantly decreased the cocooning in the silk gland of *Bombyx mori*. Furthermore, phoxim exposure significantly resulted in reductions of total protein concentrations and suppressed expressions of silk protein synthesis-related genes, including *Fib-L*, *Fib-H*, *P25*, *Ser-2*, and *Ser-3*, in the silk gland. TiO_2 nanoparticle pretreatment, however, could significantly relieve silk gland injury of *Bombyx mori*. Importantly, TiO_2 nanoparticles could remarkably elevate cocooning and total protein contents and promote expressions of *Fib-L*, *Fib-H*, *P25*, *Ser-2*, and *Ser-3* in the silk gland following exposure to phoxim.

KEYWORDS: *titanium dioxide nanoparticles, phoxim insecticide, Bombyx mori, cocooning, gene expression*

INTRODUCTION

Because of unique mechanical properties, silk from the silkworm, *Bombyx mori* (*B. mori*), has been widely used not only as a textile material, but also as a biomedical suture material for centuries, and these fibers provided important clinical repair options for many applications. However, environmental pollutants, such as pesticides, have been found to be destructive on silkworms. Non-cocooning and silk yield reduction have appeared in the sericulture as a result of the pesticide applications to cultivations, especially when mulberry trees grow next to cultivated plants in China.

Phoxim, a highly effective organophosphate (OP) pesticide, has been widely used in agriculture for pest control. The domesticated silkworm, *B. mori*, is an important economical insect that used as a source of raw silk.[1] However, *B. mori* is a nontarget organism of insecticides that is extremely vulnerable to pesticides.[2] Larvae of the silkworm may be exposed to pesticide residues via their food, the leaves of the mulberry tree (*Morus* spp.). Mulberry trees are grown in plantations and often get polluted by phoxim insecticide as a result of pesticide drift from crop fields that are adjacent to mulberry plantations.[3] Phoxim is fatal to silkworms even in tiny concentrations (ppm), which may influence their growth, reproduction, and regular physiology.[4] Moreover, pesticide poisoning leads to more than 30% reduction in silk yield each year,[5] which severely affects the healthy development of the sericulture in China.

Titanium dioxide nanoparticles (TiO_2 NPs) are widely used in various manufactured products, including sunscreen, pharmaceuticals, toothpaste, white pigment, and cosmetics, and in environmental decontamination of air, soil, and water.[6−11] A recent study showed that TiO_2 NPs played important roles in organic pollutants and act on the photocatalytic degradation of phoxim as an effective catalyst.[12] Furthermore, our previous studies found that TiO_2 NPs could enhance the metabolisms of carbohydrates and proteins of *B. mori* and fulfill the demands of necessary energy of *B. mori*,[6] increase AChE activity, remove ROS by increasing antioxidant capacity, and enhance *B. mori* survival under phoxim-induced toxicity.[13] In addition, *B. mori* larvae's resistance to BmNPV was increased by TiO_2 NP treatment and TiO_2 NPs could obviously improve the expressions of resistance-related genes that encode superoxide dismutase, catalase, glutathione peroxidase, acetylcholine esterase, carboxylesterase, and heat shock protein 21.[14]

The most interesting part of *B. mori*, however, is the silk gland for synthesizing silk proteins. Data on the study of the molecular mechanisms of silk gland injury and non-cocooning caused by phoxim exposure and the improvement of TiO_2 NP

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Table 1. Real-Time PCR Primer Pairs[a]

gene name	description	primer sequences (5′→3′)	primer size (bp)
actin3	mactin3 F	CGGCTACTCGTTCACTAC	
	mactin3R	CCGTCGGGAAGTTCGTAAG	147
Fib-L	mFib-L F	AGGTGGAAGAATCTATGAC	
	mFib-L R	TGTTGCTTTGGCTGTT	128
Fib-H	mFib-H F	ACAAGGTGCAGGAAGTGC	
	mFib-H R	AGCAATTCACACAAGGCAGT	152
P25	mP25 F	GCCCTGCTACTTGGACGAT	
	mP25 R	CGCTGACACGGCACTGAT	191
sericin 2	msericin 2 F	TCCTCAAGCACCTACACC	
	msericin 2 R	GATAGCCAATGATACACG	216
sericin 3	msericin 3 F	CTATTGTAGCCGTCCAGG	
	msericin 3 R	TTCCACCGACGAGACATG	159

[a]PCR primers used in the gene expression analysis.

pretreatment in the silk gland of *B. mori* are lacking. Therefore, we hypothesized that non-cocooning of *B. mori* following exposure to OP may be associated with the silk gland damage and decreased expressions of silk protein synthesis-related genes in the silk gland, and TiO_2 NP pretreatment may relieve this injury and increase cocooning of *B. mori* under OP-induced toxicity. Thus, the objective of the present study was to determine whether TiO_2 NP pretreatment attenuates silk gland injury and increases cocooning of *B. mori* following exposure to phoxim.

MATERIALS AND METHODS

Insect. The larvae of *B. mori* (*B. mori* L. *Qiufeng* × *baiyu*) maintained in our laboratory were reared on mulberry leaves under a 12 h light/12 h dark condition.

Chemicals. Anatase TiO_2 NPs were prepared via controlled hydrolysis of titanium tetrabutoxide. Details of the synthesis and characterization of TiO_2 NPs were described in our previous reports.[15,16] The average particle size of powdered TiO_2 NPs suspended in 0.5% w/v hydroxypropylmethylcellulose (HPMC) K4M (Sigma-Aldrich, St. Louis, MO, USA) solvent, in which the HPMC solution was prepared with deionized and distilled water after 12 and 24 h of incubation, was 5−6 nm, and the surface area of the sample was 174.8 m^2/g. The mean hydrodynamic diameter of the TiO_2 NPs in HPMC solvent was 294 nm (range: 208−330 nm), and the ζ-potential after 12 and 24 h of incubation was 7.57 and 9.28 mV.

Phoxim was purchased from Sigma-Aldrich Company.

Phoxim and TiO_2 NPs Dissolving Method. TiO_2 NPs powder was dispersed onto the surface of 0.5%, w/v HPMC, and then the solutions containing TiO_2 NPs were treated using an ultrasonic technique for 30 min and mechanically vibrated for 5 min. Phoxim was dissolved in acetone to obtain a stock solution. The stock solution was then diluted with water to obtain different concentrations for testing; 0.5% HPMC was used as the suspending agent.

Resistance Measurement. The larvae of *B. mori* maintained in our laboratory were reared on mulberry leaves under 12 h light/12 h dark conditions. According to our previous published reports, the optimum concentration of TiO_2 NPs was 5 μg/mL for growth of these larvae, and 4 μg/mL was used in subsequent experiments.[4,17] A 50 g amount of fresh mulberry leaves, *Morus albus* (L.), was dipped in 5 μg/mL TiO_2 NP suspension for 1 min and in 4 μg/mL phoxim solution for 1 min, respectively. After air-drying, TiO_2 NP-treated leaves were used to rear *B. mori* instar larvae, which were fed these leaves three times a day until the second day of the fifth-instar. Fresh leaves treated with distilled water served as controls. Phoxim-treated leaves were used to rear *B. mori* larvae, which were fed these leaves three times a day on the third day of the fifth-instar, respectively. The silkworms were then fed TiO_2 NP-treated leaves or control leaves, under a long-day photoperiod (16 h light/8 h dark) at 25 °C and approximately 75% relative humidity. Each experiment was performed three times with 500 larvae. Mortality of the larvae was recorded 36 h later. Approximately 250 larvae in each group were continuously reared to maturity; they stopped feeding and began to spin a cocoon. This was used to determine the cocooning rate of *B. mori*.

Silk Gland Collection. In 36 h after phoxim treatment, 100 fifth-instar larvae were selected randomly from each group. The larval silk glands were collected at different exposed time intervals from different treatments frozen in −80 °C for subsequent antioxidant assay.

Histopathological Evaluation of Silk Gland. For pathologic studies, all histopathological examinations were performed using standard laboratory procedures. The silk glands were embedded in paraffin blocks, and then sliced (5 μm thickness) and placed onto glass slides. After hematoxylin−eosin staining, the stained sections were evaluated by a histopathologist unaware of the treatments, using an optical microscope (Nikon U-III Multipoint Sensor System, Japan).

Assay of Total Protein Contents. A 500 mg portion of silk gland tissue was rapidly thawed and homogenized at 4 °C in 200 μL of lysis buffer (Beyotime Biotechnology Co., Jiangsu, China). Homogenates were centrifuged at 12 000*g* for 5 min at 4 °C to yield supernatants. The protein concentrations were determined by a BCA protein assay kit (Sangon Biotech, Shanghai Co., Ltd.).

Assay of Phoxim Residue Level. A 0.3 g amount of silk glands were carefully weighed and pulverized. The paste was extracted using 1 mL of ethyl acetate and homogenized in a high-speed homogenizer at 3000 r/min for 5 min. It was then centrifuged at 10 000 r/min for 10 min. The extract was dried in the oven at 45 °C. The remnant was dissolved in acetonitrile and filtered by a membrane (0.45 μm pore size). The purified sample (20 μL) was injected in the high-performance liquid chromatography (HPLC) for analysis.

Quantitative Real-Time PCR (qRT-PCR). mRNA levels of *Fib-L*, *Fib-H*, *P25*, *Ser-2*, and *Ser-3* in the silkworm glands were determined using real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR).[5,18] Synthesized complementary DNA was generated by qRT-PCR with primers designed with Primer Express Software (Applied Biosystems, Foster City, CA, USA) according to the software guidelines, and PCR primer sequences are listed Table 1.

Western Blot Analysis. The silk gland samples from the control and phoxim-exposed groups were homogenized in lysis buffer supplemented with 1 mM of PMSF. Mitochondrial and cytosolic proteins were extracted with a Mitochondrial Isolation Kit (Sangon, China) according to the manufacturer's protocol. The samples were centrifuged at 13 000 rpm for 10 min, and the supernatants were collected. The following procedure was carried out following Gu et al.[19] An antibody against α-Tubulin (Cell Signaling, USA; 1:2000) and cytochrome *c* (Genscript, China; 1:2000) was used as the primary antibody, and the HRP-conjugated goat antirabbit IgG (1:10 000) was used as the secondary antibody.

Statistical Analysis. All data were expressed as mean ± standard error of the mean (SEM). Data were analyzed by a one-way ANOVA, followed by Dunnett's *t* test for comparisons between groups. A p value < 0.05 was considered significant compared to the control.

Table 2. Effects of TiO_2 NPs on Cocooning of Phoxim-Exposed Fifth-Instar Larvae of *B. mori* after 36 h

	index			
	treatment			
	control	TiO_2 NPs	phoxim[a]	phoxim + TiO_2 NPs
rate of cocooning (%)	98.23 ± 4.91	100.00 ± 5.00	45.23 ± 2.26***[a]	95.45 ± 4.87
mean weight of cocoons (g/cocoon)	2.04 ± 0.10	2.15 ± 0.12*[a]	1.49 ± 0.07**[a]	2.00 ± 0.10
mean weight of shell layer (g/cocoon)	0.521 ± 0.026	0.558 ± 0.028*[a]	0.328 ± 0.015**[a]	0.512 ± 0.025
rate of cocoon shell (%)	25.54 ± 1.28	25.73 ± 1.29	22.04 ± 1.15*[a]	25.10 ± 1.26

[a]*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Values represent means ± SEM ($N = 5$; female silkworms or male silkworms $N = 50$ for cocooning).

RESULTS

Growth of Fifth-Instar Larvae. Symptoms of fifth-instar larvae poisoning, such as vomiting, cramps, head nystagmus, chaos climb, paralysis, and body shrink, following exposure to phoxim for 36 h were significantly observed. However, no obvious abnormalities of fifth-instar larvae were observed in the TiO_2 NPs + phoxim treated group.

Cocooning. The results concerning the effect of TiO_2 NPs on cocooning of phoxim-exposed fifth-instar larvae of *B. mori* are shown in Table 2. In spring-reared larvae, the rate of cocooning of surviving larvae was greatly reduced by phoxim exposure compared with controls ($p < 0.001$). While all larvae cocooned successfully in the TiO_2 NP pretreated groups, 95% cocooned in the combined treatments of both TiO_2 NPs and phoxim. Furthermore, TiO_2 NP treatment alone significantly increased the mean weight of the cocoons ($p < 0.05$); the mean weight of the cocoons in the phoxim-exposed groups was significantly lower than that of the controls ($p < 0.01$), whereas that from the combined treatments of both TiO_2 NPs and phoxim was higher than that of the phoxim-exposed groups ($p < 0.01$), and was as well as the controls ($p > 0.05$). The mean weight of the cocoon shell layer in the TiO_2 NP-treated groups was significantly increased ($p < 0.05$), and rate of the cocoon shell showed no marked increase as compared with the controls ($p > 0.05$). In the phoxim-exposed groups, the mean weight of the cocoon shell layer and the rate of the cocoon shell were greatly reduced ($p < 0.05$ or 0.01). In the TiO_2 NPs + phoxim treated groups, however, the mean weight of the cocoon shell layer and the rate of the cocoon shell were higher than those of the phoxim-exposed groups ($p < 0.05$ or 0.01) and were as well as the controls ($p > 0.05$). The decreased cocooning of *B. mori* caused by phoxim exposure and increased cocooning rate and total protein content of *B. mori* caused by TiO_2 NP treatment may be related to silk gland tissue injury, total protein contents, and gene expression, which are confirmed by the further histopathological observations, assays of total protein content, and gene expression in the silk gland of *B. mori*.

Histopathological Evaluation. The histological changes in the silk gland specimens are shown in Figure 1. Unexposed gland samples and TiO_2 NP-treated gland samples exhibited the normal architecture (Figure 1a,b,d), whereas those gland structures from the phoxim-exposed group were damaged; for example, epithelial cell sparseness, gland membrane crevices, and great space in the lumen were significantly observed (Figure 1c). These findings indicated that phoxim exposure led to severe damages of silk gland and TiO_2 NP pretreatment alleviated the injuries under phoxim stress.

Total Protein Contents. To explore the effect of protein contents on the cocoon of *B. mori*, the total protein concentrations in the silk glands was assayed and is exhibited in Figure 2. In the TiO_2 NP pretreated silk gland, total protein contents were increased by 24.59% ($p < 0.01$) and those of the phoxim-exposed groups and TiO_2 NPs + phoxim-exposed

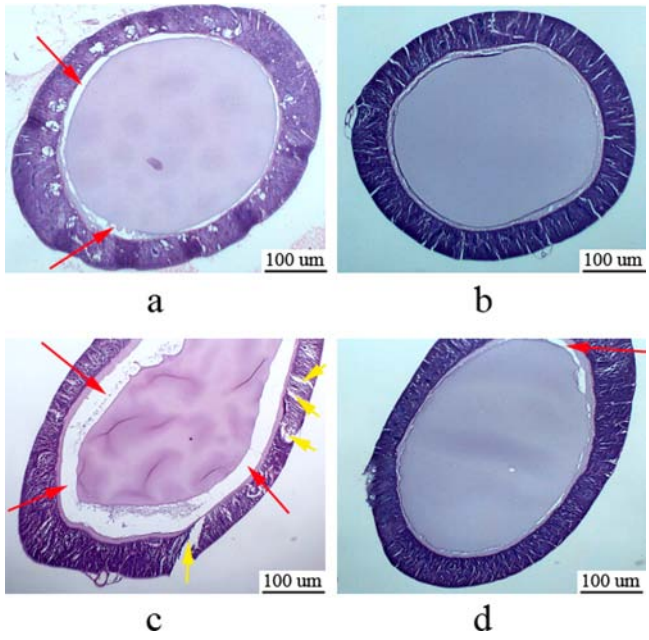

Figure 1. Histopathology of the silk gland tissue in fifth-instar larvae of *B. mori* under phoxim stress after 36 h: (a) control; (b) TiO_2 NPs; (c) phoxim; (d) TiO_2 NPs + phoxim. Red arrows indicate space in the lumen; yellow arrows indicate membrane crevice.

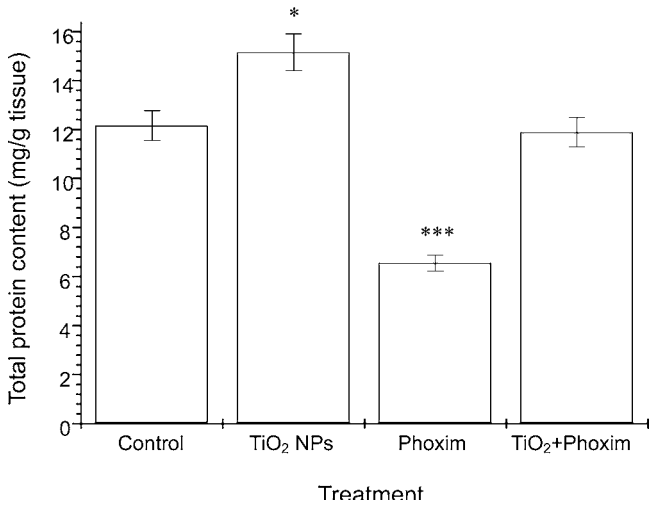

Figure 2. Effects of TiO_2 NPs on total protein contents in silk glands of phoxim-exposed fifth-instar larvae of *B. mori* after 36 h. *: $p < 0.05$; ***: $p < 0.001$. Values represent means ± SEM ($N = 5$).

Table 3. Effects of TiO_2 NPs on Gene Expression in Silk Glands of Phoxim-Exposed Fifth-Instar Larvae of *B. mori* after 36 h

	ratio of gene/actin			
	treatment			
	control	TiO_2 NPs	phoxim	phoxim + TiO_2 NPs
Fib-L	1.41 ± 0.07	9.11 ± 0.45*[a]	0.81 ± 0.04**[a]	1.27 ± 0.06
Fib-H	3.85 ± 0.19	6.01 ± 3.00**[a]	0.16 ± 0.01***[a]	3.27 ± 0.16*[a]
P25	0.67 ± 0.03	1.7 ± 0.09**[a]	0.24 ± 0.01**[a]	0.55 ± 0.03
Ser-2	0.53 ± 0.03	3.94 ± 0.20***[a]	0.23 ± 0.01**[a]	1.62 ± 0.08**[a]
Ser-3	2.24 ± 0.11	11.5 ± 0.58***[a]	0.78 ± 0.04***[a]	3.49 ± 0.17**[a]

[a]*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Values represent means ± SEM ($N = 5$).

groups were decreased by 46.05% ($p < 0.001$), and 2.22% ($p > 0.05$), respectively, as compared with the controls.

Gene Expression. To suggest the molecular mechanism of decreased cocoons and total protein content, expressions of *Fib-L*, *Fib-H*, *P25*, *Ser-2*, and *Ser-3* involved in silk protein synthesis in the silk glands of *B. mori* were measured using qRT-PCR and are exhibited in Table 3. It can be observed that the five genes' expressions in the TiO_2 NPs-treated groups were markedly elevated compared with the control, showing increases of 5.44-, 0.56-, 1.55-, 6.44-, and 4.13-fold, respectively; those from the phoxim-exposed groups were greatly suppressed as compared with the controls, showing reductions of 0.75-, 22.69-, 1.78-, 1.3-, and 1.86-fold, respectively. However, this phoxim-induced suppression was significantly relieved by TiO_2 NP pretreatment, showing increases of 0.57-, 17.89-, 1.13-, 6.04-, and 3.46-fold as compared with the phoxim-exposed groups, respectively (Table 3).

Western Blot Analysis of Fib-L. To further confirm the effects of silk protein synthesis, the level of Fib-L protein expression in the silk glands of *B. mori* was determined by Western blot analysis (Figure 3). It showed that TiO_2 NP treatment alone significantly increased Fib-L protein expression, suggesting a 21% elevation, whereas phoxim exposure led to Fib-L down-regulation as compared with the control group, suggesting a 37.89% reduction. Fib-L protein expression from TiO_2 NPs + phoxim treated groups was higher than those of the phoxim-exposed groups, showing a 16.45% increase.

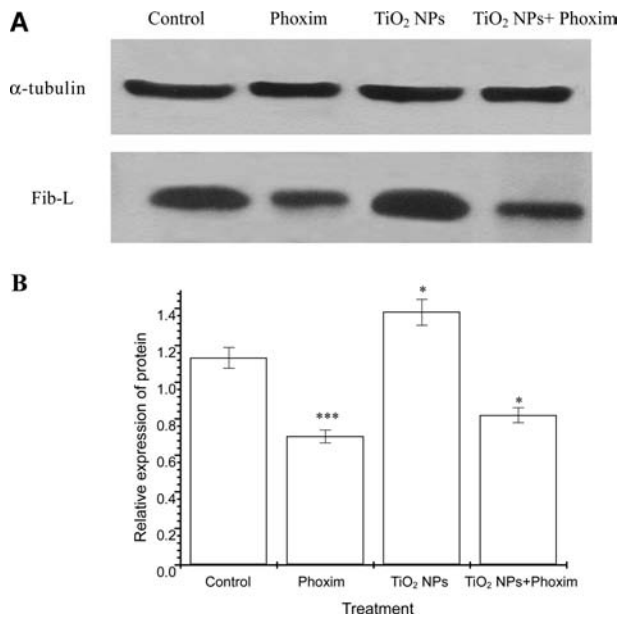

Figure 3. Effects of TiO_2 NPs on Fib-L protein expression in silk glands of phoxim-exposed fifth-instar larvae of *B. mori* after 36 h. (A) Results of Western blotting. (B) Quantitative data from scans of Western blotting. Data are expressed as fold change over control silk gland after being normalized to the total amount of α-tubulin. *: $p < 0.05$; ***: $p < 0.001$. Values represent means ± SEM ($N = 3$).

Phoxim Residue Level. TiO_2 NPs treatment can relieve the poison of phoxim; it may be the phoxim residue level in *B. mori*. Therefore, the phoxim residue levels in the silk glands were assayed by the HPLC method, suggesting 0.2 μg/g from the phoxim-exposed glands, and 0.05 μg/g from the TiO_2 NPs + phoxim treated groups, respectively. The findings indicated that TiO_2 NPs could decrease phoxim accumulation in the silk glands of *B. mori* following exposure to phoxim.

DISCUSSION

There are few studies that have focused on the prevention of insecticides on *B. mori* that deal with toxicity, retardation of development, growth, survival, and, especially, non-cocooning.[1,2,13,14] In the present article, we first investigated whether TiO_2 NP pretreatment relieves silk gland damage and increases cocooning of *B. mori* under phoxim-induced toxicity. We observed that phoxim exposure resulted in fifth-instar larvae poisoning, epithelial cell sparseness, severe membrane structure damage, and lumen vacuolization in the silk gland (Figure 1), but TiO_2 NP pretreatment could decrease larvae poisoning and repair theses damages (Figure 2). As we know, silk proteins are produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into the lumen of these glands where the proteins are stored prior to spinning into fibers. Therefore, phoxim exposure was extremely harmful to the cocooning of *B. mori*: the rate of cocooning and mean weight of the cocoons, two indices of the spinning ability of larvae, including mean weight of the cocoon shell layer and rate of the cocoon shell, were significantly reduced (Table 2). TiO_2 NP pretreatment, however, remarkably elevated levels of these parameters under phoxim stress (Table 2), which were closely related to the alterations of total protein in the silk gland (Figure 2). Moreover, the TiO_2 NP-improved silk gland structure would be helpful to both protein synthesis and storage, and elevate cocooning of *B. mori*, which may trigger the alterations of gene expressions involved in silk protein synthesis.

As we know, the silk thread spun by *B. mori* consists of two types of proteins: fibroin and sericin. The silk fibroin produced by *B. mori* is secreted from the silk gland cells as a molecular complex composing of a heavy chain (H-chain) of 350 kDa (Fib-H),[20,21] linked by disulfide linkage to two subunits: fibroin light chain (L-chain,) of 26 kDa (Fib-L) [22] and *P25*, which is a glycoprotein of about 30 kDa. [23] Our data showed that levels of *Fib-H*, *Fib-L*, and *P25* expressions were obviously reduced by

phoxim exposure and TiO_2 NP pretreatment significantly increased levels of these genes (Table 3). Furthermore, Western blotting analysis also indicated that phoxim exposure decreased Fib-L protein expression and TiO_2 NP pretreatment promoted the protein expression (Figure 3), which may lead to the alteration of the fibroin concentration in the silk gland. Sericin, the product of Ser1, Ser2, and Ser3, is a family of the adhesive silk proteins that composes a group of serine-rich proteins secreted in the midregion of the silk gland.[24,25] Sericin is categorized into many different-length proteins produced by alternatively splicing the primary transcripts of two sericin genes, Ser1 and Ser2. [26] The Ser3 protein is synthesized in the anterior portion of the middle silk gland and then appears in the outermost layer at the spinneret. [27] Results from qRT-PCR analysis demonstrated that *Ser-2* and *Ser-3* were dramatically down-regulated in the phoxim-exposed silk glands, whereas, in the TiO_2 NP-treated group, they were significantly expressed (Table 3), which may lead to the alteration of sericin protein concentration in the lumen of the middle silk gland. Taken together, the decreased total protein contents and cocooning of *B. mori* following exposure to phoxim may be closely associated with suppression of *Fib-H*, *Fib-L*, *P25*, *Ser-2*, and *Ser-3*, whereas TiO_2 NP pretreatment increased the total protein contents and cocooning via elevating expressions of *Fib-H*, *Fib-L*, *P25*, *Ser-2*, and *Ser-3* in the silk gland of *B. mori* following exposure to phoxim.

To further confirm TiO_2 NPs relieving phoxim-induced toxicity, the present article also assayed the phoxim residue level in the silk gland of *B. mori*, suggesting that TiO_2 NP pretreatment could greatly reduce the phoxim residue in the silk gland, which, in turn, led to a decrease in phoxim-induced toxicity to silkworms. However, the mechanism of decreased phoxim residue in silkworms caused by TiO_2 NP pretreatment should be studied in the future.

In conclusion, in this study, exposed *B. mori* fifth-instar larvae to phoxim caused larvae poisoning, severe structural damages in the silk gland, and significantly decreased cocooning. Non-cocooning of *B. mori* following exposure to phoxim was closely related to decreases of total protein concentrations and expressions of *Fib-H*, *Fib-L*, *P25*, *Ser-2*, and *Ser-3* in the silk gland. TiO_2 NP pretreatment, however, could significantly relieve larvae poisoning, repair silk gland damages, and increase cocooning under phoxim-induced toxicity. The increases of cocooning of *B. mori* by TiO_2 NP pretreatment may be closely associated with increased total protein concentrations, expressions of *Fib-H*, *Fib-L*, *P25*, *Ser-2*, and *Ser-3* in the silk gland, and low phoxim residue level. Therefore, our findings suggest that TiO_2 NP pretreatment could relieve phoxim toxicity to *B. mori* and promote cocooning, which would be used in the sericulture in the future.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on December 5, 2013, with an error to Figure 3. The corrected version reposted with the issue on December 18, 2013.