Molecular Signatures of Reduced Nerve Toxicity by CeCl₃ in Phoxim-exposed Silkworm Brains

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CeCl₃ can reduce the damage caused by OP pesticides, in this study we used the brain of silkworms to investigate the mechanism of CeCl₃ effects on pesticide resistance. The results showed that phoxim treatments led to brain damages, swelling and death of neurons, chromatin condensation, and mitochondrial damage. Normal nerve conduction was severely affected by phoxim treatments, as revealed by: increases in the contents of neurotransmitters Glu, NO, and ACh by 63.65%, 61.44%, and 98.54%, respectively; decreases in the contents of 5-HT and DA by 53.19% and 43.71%, respectively; reductions in the activities of Na⁺/K⁺-ATPase, Ca²⁺/Mg²⁺-ATPase, and AChE by 85.27%, 85.63%, and 85.63%, respectively; and increase in the activity of TNOS by 22.33%. CeCl₃ pretreatment can significantly reduce such damages. Results of DGE and qRT-PCR indicated that CeCl₃ treatments significantly upregulated the expression levels of CYP4G23, cyt-b5, GSTs-σ₁, ace₁, esterase-FE₁₄, and β-esterase 2. Overall, phoxim treatments cause nerve tissue lesions, neuron death, and nerve conduction hindrance, but CeCl₃ pretreatments can promote the expression of phoxim resistance-related genes in silkworm brains to reduce phoxim-induced damages. Our study provides a potential new method to improve the resistance of silkworms against OP pesticides.

Silkworm (Bombyx mori, B. mori) is an important economic insect and a model species for Lepidoptera, in China it produces more than 80% of raw silk of the world¹. It has been domesticated for about 5,700 years in China. However, B. mori became very sensitive to pesticides and other chemicals because of long-term indoor breeding and limited exposure to the outside environment. Therefore, it is also used as a model insect to study the toxicology of pesticides and pest control and serves as an environmental indicator. Organophosphorus (OP) based pesticides are one of the most widely used pesticides. The main mechanism of action of OP pesticides is to irreversibly bind acetylcholinesterase (AChE) and inhibit its activity, which leads to the accumulation of neurotransmitter acetyl choline (ACh) in synaptic clefts then insect convulsion and eventual death, because nervous excitement cannot be terminated²,³. Wide use of pesticides has significantly improved agriculture productions, but the pollution from them to the environment has also become a big problem. In China, pesticide-contaminated mulberry causes up to 30% loss in silk industry every year⁴. Therefore, how to reduce such losses has become a popular topic in recent years. Previous studies have shown that titanium dioxide can reduce the damage caused by OP pesticide phoxim, and the mechanism has been explored⁵.

Rare earth elements (REEs) are widely used in various industries due to their diverse physical and chemical properties⁶, including agriculture and pharmaceutical industry, for example Cerium can relieve the inhibition of chlorophyll biosynthesis of maize caused by magnesium deficiency⁷. Cerium can increase taxol accumulation of Taxus cuspidata cells⁸. Recent study showed that CeCl₃ pretreatment could increase the growth and survival rate of B. mori under phoxim-induced toxicity by increasing

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antioxidant capacity and improving protein and carbohydrate metabolism. CeCl₃ pretreatment could decrease oxidative damage of B. mori caused by nucleopolyhedrovirus infection via increasing antioxidant capacity. CeCl₃ pretreatment could alter the gene expression pattern and relieves the damages in the midgut of B. mori caused by phoxim. CeCl₃ can relieves the damages caused by phoxim, protect B. mori silk gland and remit the reduction in body weight and cocooning rate through changing gene expression patterns and reducing oxidative stress.

Brain is the main part of central nervous system and the important target of phoxim. Whether CeCl₃ can relieve the brain damage in B. mori caused by phoxim is still unknown. In this study, brain was used as the study object to further explore the mechanism of relief from toxic symptoms caused by phoxim under CeCl₃ pretreatment.

Results

Histopathological evaluation of brain. As shown by the histological photomicrographs of B. mori larval brain sections in Fig. 1, both control group (Fig. 1A) and CeCl₃-treated group (Fig. 1B) had no abnormal pathological changes, with spherical glial cells, fusiform neurons cells and nerve fiber tracts showing clear and complete structures. In the phoxim-exposed group, we observed that glial cells and neurons were swollen, along with loss in cell contents, nucleus fragmentation, cell death, nerve fibers breakage, protein aggregation, and adipose degeneration (Fig. 1C). As a contrast, the CeCl₃ + phoxim-treated group did not show such pathological changes (Fig. 1D). These results demonstrated that phoxim exposure caused brain damages, while CeCl₃ treatments were able to reduce them.

Brain ultrastructure evaluation. Changes in brain ultrastructure in silkworms were presented in Fig. 2. Both the control group (Fig. 2A) and CeCl₃-treated group (Fig. 2B) had normal structures, along with evenly distributed nuclear chromatin, integral mitochondria structure, clear mitochondria ridges. The phoxim-treated group (Fig. 2C), on the other hand, showed karyopyknosis, chromatin marginalization, swelling mitochondria, vacuolar degeneration, rough surfaced endoplasmic reticulum, and extended golgi's apparatus, while the CeCl₃ + phoxim-treated group had reduced phoxim-induced damages (Fig. 2D).
Neurotransmitter contents and enzyme activities in the brain. Contents of neurotransmitters and activities of related enzymes were analyzed in order to investigate phoxim-induced nerve damages in the brain of fifth instar larvae. As shown in Table 1, in the control group and CeCl₃-treated group, the contents of neurotransmitters Glutamate (Glu), nitric oxide (NO), ACh, 5-hydroxytryptamine (5-HT), and dopamine (DA) did not change significantly. However, in the phoxim-treated group, contents of Glu, NO and ACh were all significantly increased, by 63.65%, 61.14%, and 98.54%, respectively (P < 0.01), while the 5-HT and DA contents were significantly decreased by 53.19% and 43.71%, respectively (P < 0.01) (Table 1). These results indicated that CeCl₃ treatments significantly alleviated phoxim-induced damages. In addition, the CeCl₃-treated group did not show significant differences in the activities of Na⁺/K⁺-ATPase, Ca²⁺/Mg²⁺-ATPase, AChE, and total nitric oxide synthase (TNOS) from those of the control.

Table 1. Effects of phoxim and CeCl₃ on neurotransmitter contents in brain of silkworm. *P < 0.05, and **P < 0.01. Values represent means ± SD (N = 5).

|          | GLu (µmol/g protein) | NO (µmol/g protein) | ACh (µg/g tissue) | 5-HT (µg/g tissue) | DA (µg/g tissue) |
|----------|----------------------|---------------------|-------------------|-------------------|-----------------|
| Control  | 55.35 ± 1.22         | 72.32 ± 1.49        | 10.26 ± 1.91      | 23.35 ± 2.91      | 20.11 ± 1.83    |
| CeCl₃    | 52.49 ± 1.26         | 67.53 ± 1.17        | 8.55 ± 1.39       | 25.82 ± 3.03      | 22.25 ± 2.15    |
| Phoxim   | 90.58 ± 1.82**       | 116.54 ± 2.31**     | 20.37 ± 2.86**    | 10.93 ± 0.91**    | 11.32 ± 1.28**  |
| Ce+Phoxim| 67.12 ± 1.45*        | 79.89 ± 1.85        | 12.21 ± 1.66      | 20.71 ± 1.21      | 18.9 ± 1.69     |

Figure 2. Ultrastructure of the brain tissue in fifth-instar larvae after phoxim exposure. (A) Control; (B) CeCl₃; (C) phoxim; (D) CeCl₃ + phoxim. Green arrows indicate karyopyknosis and chromatin marginalization, blue arrows show mitochondria swelling and became deformed, crest broken.
The group of CeCl₃

With CeCl₃

These genes are involved in nerve conduction, pesticide metabolism, apoptosis, and oxidative stress. Different expression patterns in DGE assay were selected for qRT-PCR verification due to their functions.

A number of genes that showed significantly different expression patterns in DGE assay were selected for qRT-PCR verification due to their functions. These genes are involved in nerve conduction, pesticide metabolism, apoptosis, and oxidative stress. With CeCl₃ + phoxim treatments, the expression levels of cytochrome P450 family 4G23 (CYP4G23), cytochrome b5 (cyt-b5), GSTs-α1, acetylcholinesterase type 1 gene (ace1), esterase-FE4, β-esterase 2, and catalase (CAT) were increased 12.583, 8.623, 16.462, 5.843, 6.714, 2.583, and 2.793 fold, respectively. With CeCl₃ treatment, CYP4G23 was increased by 3.472 fold, while phoxim treatment increased CYP4G23’s level by 6.363 fold, ace1’s level by 19.453 fold, and esterase-FE4’s level by 3.671 fold (Table 3). The results indicated that CeCl₃ changes the gene expression response of silkworm larva’s brains to phoxim, consistent with the DGE results.

Discussion

OP pesticides are widely used in China. Application of large quantity of pesticides can certainly control varied types of pests, but it is also polluting the environment. Silkworms are very sensitive to pesticides, thus pesticide pollution causes serious losses to China’s sericulture. How to increase silkworm’s pesticide resistance to mitigate economic losses has become an urgent problem. In this study, we found that addition of an appropriate amount of CeCl₃ onto mulberry leaves can reduce phoxim-induced damages. The mechanism of CeCl₃’s effects on phoxim-induced damages was also investigated in order to seek for methods to better improve silkworm’s resistance to pesticides.

The major damage of phoxim comes from its destroy to nervous 3. We also performed detailed investigation on silkworm brains after phoxim treatments. Phoxim caused serious brain tissue damages and cell death (Fig. 1). Analysis of ultrastructure revealed severely damaged endoplasmic reticulum, golgi apparatus, and mitochondria (Fig. 2). At the same time, the contents of neurotransmitters Glu, NO, and 5-HT were increased significantly, along with significantly decreased contents of DA and significantly inhibited activities of Na⁺/K⁺-ATPase, Ca²⁺/Mg²⁺-ATPase, and AChE. Na⁺/K⁺-ATPase, Ca²⁺ and Mg²⁺ ion, AChE play an important role in conduction of action potential, ACh transport, ACh degradation respectively. When CeCl₃ was given to the larvae, such changes were significantly relieved.

Cytochrome P450, GSTs, and esterase are essential parts of the detoxification system of insects. In this study, we observed increased expression of CYP4G23, a member of CYP4 family which are well consistent with their predicted role in xenobiotic metabolism, indicating that phoxim poisoning induces CYP4G23 as a response in larval brain, such response was even stronger in the CeCl₃ pretreatment group (Table 3). Cyt-b5, an essential molecule in P450-mediated detoxification, was significant increase in the group of CeCl₃ + phoxim (Table 3). GSTs have multiple subunits and play important roles in the biotransformation of exogenous compounds, drug metabolism, and the protection against peroxidation.

### Table 2. Effects of phoxim and CeCl₃ on enzyme activities in 5th-instar larva brain of silkworm.

| Group       | Na⁺/K⁺-ATPase (U/mg protein·min) | Ca²⁺/Mg²⁺-ATPase (U/mg protein·min) | AChE (U/mg protein·min) | TNOS (U/mg protein·min) |
|-------------|----------------------------------|-------------------------------------|-------------------------|-------------------------|
| Control     | 2.58 ± 0.31                      | 1.74 ± 0.12                         | 1.74 ± 0.19             | 7.22 ± 0.49             |
| CeCl₃       | 3.39 ± 0.39*                     | 1.82 ± 0.17                         | 1.95 ± 0.21             | 6.73 ± 0.38             |
| Phoxim      | 0.38 ± 0.08**                    | 0.23 ± 0.07***                      | 0.25 ± 0.06***          | 11.64 ± 1.15**          |
| Ce+Phoxim   | 1.90 ± 0.13*                     | 1.42 ± 0.15*                        | 1.48 ± 0.15*            | 7.98 ± 0.61             |

*P < 0.05, **P < 0.01, and ***P < 0.001. Values represent means ± SD (N = 5).
damages. Therefore, increases in the expression of GSTs are one of the major reasons of enhanced pesticide resistance\(^{19}\). In this study, we found that the subunit GSTs-\(\sigma\)1’s expression was significantly reduced by phoxim but significantly increased by CeCl\(_3\) pretreatments (Table 3), indicating that CeCl\(_3\) promotes the response to phoxim through increasing GSTs contents. Esterases exert their detoxification effects by direct binding to or hydrolysis of pesticides, and many studies have reported that overexpression of esterases can increase insects’ resistance to pesticides\(^{20,21}\). The overexpression of \(\beta\)-esterase increase

**Figure 3. Significantly changed genes classify.** (A) Functional categorization of 355 genes which significantly altered by CeCl\(_3\) pretreatment; (B) Functional categorization of 282 genes which significantly altered by phoxim exposure; (C) Functional categorization of 422 genes which significantly altered by CeCl\(_3\) + phoxim treatment; Genes were classified based on the GO function.
Myzus persicae esterase-FE4 is positive correlation to acephate in (SULZER)23. In the present study, CeCl3 use CeCl3 as an additive to improve silkworms’ pesticide tolerance in sericulture.

These complexes can also reduce the chlorpyrifos and parathion residue in jujube 34. plexes with saccharides and revealed that the complexes were able to degrade methamidophos, omethoate and dimethoate33. These complexes can also reduce the chlorpyrifos and parathion residue in jujube 34. Mitochondria are not only the energy factories of eukaryotic cells but also the primary targets of toxicity after pesticides enter cells27-30. In the present study, phoxim caused serious damages to the mitochondria of silkworm larval brains, cell abnormalities, and even apoptosis, and all these damages were alleviated by CeCl3 pretreatments (Figs 1 and 2). Results from high-throughput sequencing indicated that CeCl3 treatment can increase the expression of CAT, xanthine dehydrogenase (XDH), and serine-pyruvate ami-notransferase (AGT) (Table 3, Table S1), these enzymes reduce mitochondrial damages through scav-enging free radicals.

Cerium ion has some special features, including functioning as an antibiotic31 and cleaving the phosphodiester of DNA32. Wang et al. attempted to hydrolyze the phosphodiester of OP using Cerium complexes with saccharides and revealed that the complexes were able to degrade methamidophos, omethoate and dimethoate33. These complexes can also reduce the chlorpyrifos and parathion residue in jujube34. We speculate that absorbed Cerium ion may hydrolyze phoxim to some extent and protect the silkworm, which needs further studies to confirm.

In this study, we found that CeCl3 pretreatment could reduce phoxim-induced nerve damages. CeCl3 enhanced the expression of detoxification enzymes, esterases, P450s, and GSTs, which participate in phoxim metabolism; CeCl3 could also upregulate the genes related to free radical clearance and decrease the expression of apoptosis genes to protect neurons in the brain. As a result, CeCl3 may enhance phoxim metabolism in silkworms to protect their nerve systems. This study illustrated the potential mechanisms of CeCl3’s effect to enhance phoxim metabolism in silkworm brains and provided a theoretical basis to use CeCl3 as an additive to improve silkworms’ pesticide tolerance in sericulture.

### Methods

**Insect and chemicals.** The larvae of *B. mori* (strain: Qiufeng × baiyu) maintained in our laboratory were reared at 27 ± 2°C on mulberry leaves under a 12 h light/12 h dark cycle.

Phoxim was purchased from Sigma-Aldrich (USA). CeCl3 (analytical grade, 99.99%) was purchased from Shanghai Chem. Co. (China).

**Treatment and brain tissue collection.** Phoxim stock solution was prepared by 10 × dilution using acetone. For the treatment, phoxim stock solution was dissolved in water to obtain a concentration of 4 μg/mL. The lethal concentration (LC50) of phoxim in *B. mori* was 7.86 μg/mL, and at 4 μg/mL, *B. mori* showed poisoning symptoms without death14. In a pre-experiment, different concentrations (0.1, 0.2, 0.5, 1.0, and 1.5 mg/L) of CeCl3 was used, which were administered to fifth-instar larvae, the optimum concentration of CeCl3 was 0.5 mg/L for growth of these larvae10. *B. mori* larvae were fed with CeCl3-treated leaves (leaves were dipped in 0.5 mg/L CeCl3 solution for 1 min and dried in the air) and normal leaves respectively three times a day until the 2nd day of fifth-instar. Then a part of the larvae of these two groups were feed with phoxim-treated leaves (leaves were dipped in 4 μg/mL phoxim for 1 min and dried in the air). Each treatment was performed three times. Forty-eight hours after phoxim

| Gene       | Treatment                                      | phoxim/Control | CeCl3/Control | CeCl3 + phoxim/Control |
|------------|-----------------------------------------------|----------------|---------------|------------------------|
|            | qRT-PCR (Fold)                  | DGE (log, value) | qRT-PCR (Fold) | DGE (log, value) |
| CYP4G23    | 3.472*** 5.537                     | 6.363*** 4.228  | 12.583*** 5.755 |
| Cyt-b5     | 1.047 No difference                | 0.945 No difference | 8.623*** 4.854 |
| GST-σ1     | 0.835 −0.408                       | 0.262** −2.827  | 16.462*** 4.002 |
| accl       | 0.908 0.072                        | 19.453*** 0.956 | 5.843*** 1.059 |
| esterase-FE4| 1.142 No difference               | 2.671** 5.365   | 6.714*** 5.487 |
| β-esterase 2| 0.722 No difference                | 0.869 No difference | 2.583** 2.209 |
| CAT        | 1.106 No difference                | 1.028 No difference | 2.793** 2.998 |

Table 3. Comparison between fold-difference with qRT-PCR results and DGE assay in each group.

*P < 0.05, **P < 0.01, and ***P < 0.001. Values represent means ± SD (N = 5).
treatments, 100 fifth-instar larvae was selected randomly from each group to collect brain tissues that were frozen at $-80^\circ C$ for further study.

**Histopathological evaluation of brain.** All histopathological examinations were performed using the follow laboratory procedures. Five brains from the larvae of each group were embedded in paraffin, sliced (5 μm thickness), placed onto glass slides, and stained with hematoxylin-eosin for 15 min. Stained samples were observed and photographed using an optical microscope (Nikon U-III Multi-point Sensor System, Japan).

**Observation of brain ultrastructure.** Five larvae's brain tissues of each group were fixed in freshly prepared 0.1 M sodium cacodylate buffer with 2.5% glutaraldehyde and 2% formaldehyde, before being treated at 4 °C with 1% osmium tetroxide in 50 mM sodium cacodylate (pH 7.2-7.4) for 2 h. Staining was performed overnight with 0.5% aqueous uranyl acetate. After serial dehydration with ethanol (75, 85, 95, and 100%), the specimens were embedded in Epon 812 and sliced. Ultrathin sections were treated with uranyl acetate and lead citrate, and observed with a HITACHI H600 TEM (HITACHI Co., Japan). The damages of brain was determined by observing the changes in nuclear morphology, (e.g., chromatin condensation and fragmentation).

**Assay of enzymatic activities.** To determine enzymatic activities, brain tissues were homogenized in 0.15 M NaCl. The homogenate of brains was centrifuged at 3,000 g for 15 min at 4 °C. A portion of supernatant was used to measure the activities of different enzymes. The activities of AChE, Ca$^{2+}$-ATPase, Ca$^{2+}$/Mg$^{2+}$-ATPase, Na$^{+}$/K$^{+}$-ATPase, and TNOS in the brain were measured spectrophotometrically with commercial kits (Nanjing Jiancheng Bioengineering Institute, China), targeting the oxidation of oxyhaemoglobin to methaemoglobin by nitric oxide.

**Measurements of neurochemicals.** The homogenate of brains was centrifuged at 12,000 g for 20 min at 4 °C. The concentrations of DA, 5-HT, and ACh were measured spectrophotometrically with commercially kits (Nanjing Jiancheng Bioengineering Institute, China).

Glu concentrations were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China), and standard curves were generated by using standard Glu stock solutions. Sample Glu levels were detected using a spectrophotometer at 340 nm and expressed as μmol/g prot. The concentration of NO in the brain was measured using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China). The OD value was determined by using a spectrophotometer (U-3010, Hitachi, Japan). NO results were read with OD values at 550 nm. The results were calculated using the following formula: NO ($\mu$mol/L) = ($A_{sample} - A_{blank}$)/($A_{standard} - A_{blank}$) × 20 ($\mu$mol/L).

**Total RNA isolation.** Trizol reagent was used to extract the total RNA from brain samples (Takara, Dalian China) and treated with DNase to remove potential genomic DNA contamination. The quality of the RNA was quantitated spectrophotometrically at 260 and 280 nm.

**Digital gene expression library preparation and sequencing.** For RNA library construction and deep sequencing, equal quantities of brain RNA samples (n = 3) were pooled for the control group and the treatment group, respectively. Approximately 6μg of RNA representing each group was submitted to Solexa (now Illumina Inc.) for sequencing. Detailed methodology was according to the method described by Gu et al.35.

**qRT-PCR analysis.** The specific primers for the 7 genes of interest are listed in Table 4. The internal reference gene was actin3. qRT-PCR was performed using the 7500 Real-time PCR System (ABI) with

| Gene     | Primer sequences (5’-3’)          | Product size (bp) |
|----------|----------------------------------|-------------------|
| CYP4G23  | TATGGACACGCCCATAAAAG GTAGGAGATTGGGTGTTGC | 119              |
| Cyt-b5   | CTGAAGCAGAAGACCGCAC TATCGCCACCAGCCAGATG | 135              |
| GSTs-σ1  | TGGAGTTCTCGATGATAT CTTTGTGCTCAGACTATC | 150              |
| act1     | CTCCAGTTCAAGGTCGTTGCG ACAGTGCTGTCGCTGTAAG | 197              |
| EST-FF4  | GTGGCACTCTTGGGTGGAG CTTTGTGCTGATAG | 130              |
| β-esterase 2 | ACCCAATACATACCAGCTG CACAAAGGACATAGAGCC | 135              |
| CAT      | AATCTTCTCAGAGCCAGAC AGATAAACGACAGACCACATC | 109              |
| Action3  | CGGCTACTCGTTCACACC CCGTGAGGAAGTTGTAAG | 147              |
SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer’s instructions. The qRT-PCR analysis was carried out following the method described in the studies of Peng et al. and Wang et al.3,6.

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Author Contributions
Binbin Wang, Fanchi Li and Min Ni, wrote the main manuscript text, Hua Zhang, Kaizun Xu, Jianghai Tian and Jingsheng Hu prepared Table S1 and Fig. 3, Weide Shen and Bing Li designed the experiments. All authors read and approved the final manuscript.

Additional Information
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