Mechanisms of the Toxicity of Chiral Pesticides Dinotefuran Enantiomers on Esiena fetida Earthworm

Yaofang Zhang[1][a], Youpu Cheng[1][b], Yu Cheng[1,2,][c], Lijuan Lv[1][d], Yanling Xu[1][e] and Chengling Gao[1][f]

[1]*Tianjin Agricultural University, No. 22 Jinjing Road, Tianjin, China*

[2]*Tianjin Institute of Agricultural Quality Standard and Testing Technology, Jinjing Road, Tianjin, China*

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Abstract: As a promising insecticide, dinotefuran has been commercialized and widely used around the world. In this study, the acute toxicity of Rac-dinotefuran and its two enantiomers on earthworm were estimated by artificial soil method according to the OECD criteria at the individual-tissue-cell-molecule level. The 14d-LC50 values were 2.372 mg/kg for Rac-(±)-dinotefuran, 1.158 mg/kg for S-(+)-Dinotefuran and 6.002 mg/kg for R-(-)-Dinotefuran respectively. E. foetida was exposed to different concentrations of Rac-dinotefuran and its two enantiomers and the enzyme activities, DNA damage and gene expression were measured on 3, 7, 14, 21, and 28 days of post treatments, respectively. The results showed that Rac-dinotefuran and its two enantiomers caused obvious modulations on DNA damage, enzyme activities and gene expression. Additionally, the toxicity of Rac-dinotefuran and its two enantiomers behaved in time-dose-dependent manner.

1 INTRODUCTION

Neonicotinoid pesticides (NNs) are nitroguanidine systemic insecticides that are commonly used to protect seedling from leaf feeding by early season pests (Jeschke, 2008). Because of the high selectivity, high efficiency and low toxicity to mammals, neonicotinoid insecticides are now the most widespread used pesticides around the world. (Morrissey, 2015, Sparks, 2015). Neonicotinoid insecticides including imidacloprid, clothianidin, thiamethoxam, acetamiprid, and dinotefuran are among the most effective insecticide recently introduced to control pest with novel modes of action (Lina, 2012). As the third generation neonicotinoid insecticide, dinotefuran is deemed to be a promising insecticide with emproved chemical and biological properties, such as wide spectra of targets, high insecticidal efficacies, and environment safety (Hem, 2012). Given the differences of chiral pesticides enantiomers in the bio-activities, toxicities, and environmental behavior, many researchers have been vigilant to the security of chiral pesticides (Qi, 2015). However, dinotefuran inevitably permeates into the natural environment and sap the quality of soil. Although, the toxicity of some pesticides can be partially and slowly mitigated by some abiotic factors, such as degradation, migration and transformation, the contamination remains a long-lasting problem since the pesticides including dinotefuran are notoriously clumsy to be completely purged (Morrissey, 2015).

Therefore, the acute toxicity of Rac-dinotefuran and its two enantiomers on earthworems were studied. Meanwhile, we compared the changes of enzyme activities, DNA damage levels and the modifications of gene expression of *E.*foetida with and without the pesticides. The results provided scientific basis for an evaluation of the environmental safety on soil ecosystems.

[a] https://orcid.org/0000-0001-7677-9207
[b] https://orcid.org/0000-0002-0171-0165
[c] https://orcid.org/0000-0002-8607-9981
[d] https://orcid.org/0000-0002-3217-7535
[e] https://orcid.org/0000-0001-6190-9566
[f] https://orcid.org/0000-0001-5057-780X

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2 ATERIALS AND METHODS

2.1 Materials and Reagents

Earthworms (*E.* fetida) were purchased from an earthworm cultivating farm (Tianjin, China). Dinotefuran (98.0%) were provided by the ministry of agriculture pesticide identification. R-(-)-dinotefuran and S-(+)-dinotefuran were prepared in our laboratory. The experimental soil was artificial soil and prepared according to the method described in the OECD guideline (OECD, 2014).

2.2 Acute Toxicity Testing using Earthworm E.fetida

The acute toxicity was conducted according to the OECD standard method (OECD, 2014). For the media lethal concentrations (LC_{50}) of Rac-(±)-Dinotefuran and its enantiomers caculation, seven test concentrations were used, Rac-(±)-Dinotefuran (0.829, 1.077, 1.401, 1.821, 2.367, 3.077 and 4.000 mg/kg), S-(+)-Dinotefuran (0.491, 0.638, 0.829, 1.077, 1.401 1.821 and 2.367 mg/kg) and R-(-)-Dinotefuran (2.072, 2.693, 3.501, 4.552, 5.917, 7.692 and 10.000 mg/kg). Each concentration contained ten healthy earthworm and with three replicates.

2.3 Experimental Design

Based on the acute toxicity experiment results, three different concentrations (0.1, 0.5 and 1.0 mg/kg) and three replicates for each concentration with artificial soil were used in the present study. The control groups were prepared similarly but without insecticide. There are 0.5 g of dry cow dung was added onto the artificial soil surface weekly from days 1 to 28 and the same dose of artificial soils were replaced on the days of 14. All the treatments were cultured at 20±1 ℃ in 80 %-85 % relative humidity for 16 h in light and 8 h in the dark, and five exposure periods (3, 7, 14, 21 and 28 d) were tested. Each earthworm was washed with distilled water, gently dried with absorbent paper, and stored at -80 ℃ before analysis.

2.4 Determination of Enzyme Activities

One Gut-cleaned earthworm was randomly selected and homogenized in 100 mM phosphate buffer (pH 7.2). The supernatant was collected after centrifuging at 10000 rpm for 30 min (at 4 ℃). SOD activity was tested according with the method of Song et al. (Song, 2009). CAT activity was determined by measuring the consumption of H_2O_2 (Xu, 1997). POD activity was determined using the method of Kochba et al. (Kochba, 1977).

2.5 Comet Assay

Earthworm coelomocytes were performed as Eyambe et al. Described (Eyambe, 1991). The comet assay was described by the method of Mahsa et al. (Mahsa, 2014), which was used to determine the degree of DNA damage. After electrophoresis, each slide was neutralized with neutralizing buffer every 5 min for 3 times, dehydrated with 95 % ethanol and stained with SYBR green. At last, the slides were observed under fluorescence microscope (Olympus, BX51, Japan).

2.6 Real-time PCR Analysis

Total RNA was obtained using the Total RNA extraction kit and reverse-transcribed to first-strand cDNA was performed using the PrimeScript™ RT reagent Kitr. The synthesized cDNA was stored at -80 ℃ prior to use for real-time PCR. TransStart Top Green qPCR SuperMix was used in real-time PCR experiments, which was performed on a real-time PCR system. The expression of five target genes (SOD, MT, HSP70, TCTP) were compared to the expression of the housekeeping gene (β-actin) and presented as relative gene expression compared to the control. The relative gene expression level was calculated using the $2^{-\triangle\triangle Ct}$ method (Lukkari, 2004).

2.7 Statistical Analysis

Each treatment was analyzed with three replicates. The data were analyzed with SPSS 17.0 statistical software and the results were presented as means ± standard deviation (SD). The comet images were analyzed using CASP software. Olive tail moment (OTM) value was used to determine the degree of DAN damage.

3 RESULTS AND DISCUSSION

3.1 Acute Toxicity

After 14 days exposure, no mortality was observed in control group. Dinotefuran and its enantiomers showed different degree of toxicity on earthworms. The values of 14d-LC_{50} were Rac- (±)-Dinotefuran 2.372 mg/kg, S-(+)-Dinotefuran 1.158 mg/kg and R-(-)-Dinotefuran 6.002 mg/kg. The results showed that Rac-dinotefuran and its enantiomers are moderately toxic to *Eisenia foetida*. The data showed that the mortality is both concentration-dependent and application time-dependent for all insecticides test. The acute toxicity is S-(+)-Dinotefuran>Rac-(±)-Dinotefuran>R-(-)-Dinotefuran.

3.2 Enzyme Activities Assay

As antioxidant enzymes, SOD, CAT and POD, play an important role in scavenging excess reactive oxygen species (ROS) and promoting the growth of healthy cells (Ye, 2016). SOD plays a key role in decomposing $O_2^{\cdot-}$ to H_2O_2 and O_2, which is thought to be the first line to prevent the harm of ROS (Liu, 2016). CAT can decompose H_2O_2 to H_2O and O_2, which is considered to play an important role in detoxification of free radicals derived from oxygen. As one type of redox enzyme, POD can eliminate H_2O_2 and other organic hydroperoxides to protect the body from the damage posed by ROS (Niu, 2013). The possible biochemical effects of dinotefuran and its two enantiomers on the activities of SOD, CAT and POD in *E.foetida* were measured on the 3th, 7th, 14th, 21th, and 28th day respectively.

As is shown in Figure 1(A, B and C), After 7 days exposure, the SOD and CAT activities showed the same trend of variation under high concentration of S-(+)-Dinotefuran treatment groups which showed significant pre-inhibiting and post-activating effects. Their results suggested that a high concentration of S-(+)-Dinotefuran may induce excess toxicity which inhibited activities of SOD, CAT and POD. At the same time, a high concentration of S-(+)-Dinotefuran can induce oxidative stress and induce the expression of antioxidant enzymes to overcome the stress caused by pollution. Finally, the SOD and CAT exhibited higher activities, resulting in eliminating the excessive ROS production. However, the POD activities in the high concentration of S-(+)-Dinotefuran treatment groups were inhibited in the whole exposure time (P<0.001). Maybe the toxicity is stronger to POD than to other antioxidant enzymes. After 28 days of exposure, the SOD, CAT and POD activities in 1 mg/kg and 0.5 mg/kg S-(+)-Dinotefuran treatment groups changed more significantly than the other groups and exhibited dose-dependent elevate effect. The results showed that the S-(+)-Dinotefuran is more toxic than Rac-(±)-Dinotefuran and R-(-)-Dinotefuran.

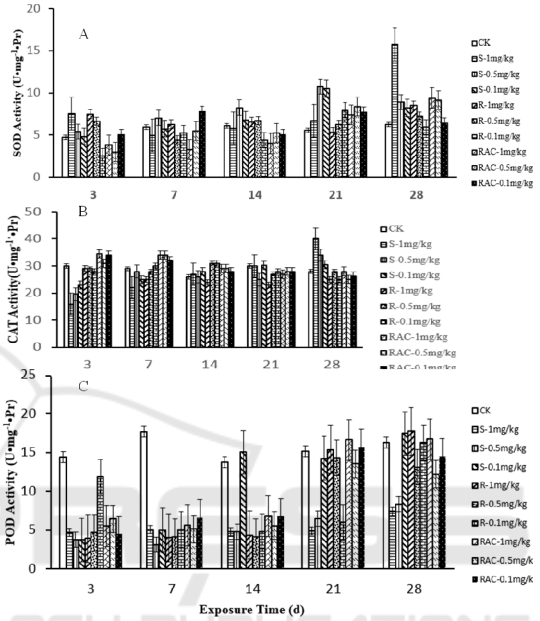

Figure 1: The effect of dinotefuran and its enantiomers on SOD (A), CAT (B) and POD (C) activity of *Eisenia foetida.* Data are described as mean±SD (n=3). Statistical significance compared with controls: $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001.

3.3 DNA Damage Induced by Dinotefuran and Its Enantiomers

The genotoxicity of dinotefuran and its enantiomers to *Eisenia foetida* were evaluated by comet assay. As is shown in Figure 2, after 3 d treatments, there were no significant difference in OTM values, indicating that after transit exposure did not cause DNA damage. On the 7th day, the OTM values of the S-(+)-Dinotefuran and 1 mg/kg Rac-(±)-Dinotefuran treatment groups were significantly higher than other treatment groups and control group (P<0.001, P<0.05). After 14 d treatments, a significant increase in the OTM values was observed for 1 mg/kg treatment groups and the OTM values increased with the increase of dosage and exposure time. The OTM values are

S-(+)-Dinotefuran>Rac-(±)-Dinotefuran>R-(-)-Dinotefuran. However, the OTM did not change significantly with the treatments of the low concentration of dinotefuran and its enantiomers compare the 21th day. The study indicated that the DNA was damaged significantly by high concentrations (1 mg/kg and 0.5 mg/kg) of exposure to dinotefuran and its enantiomers and the toxicity was S-(+)-Dinotefuran > Rac-(±)-Dinotefuran > R-(-)-Dinotefuran.

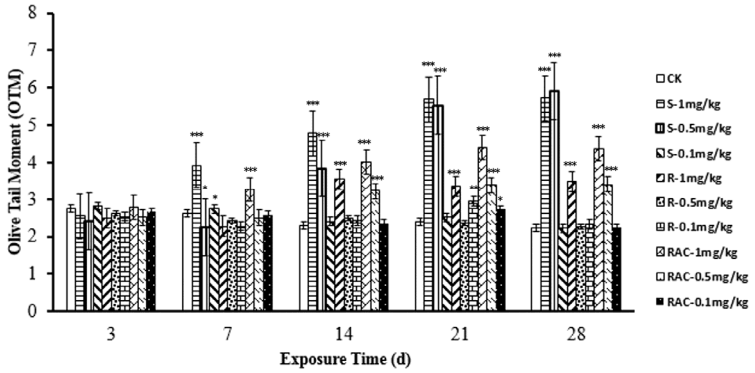

Figure 2: The effect of dinotefuran and its enantiomers on DNA damage degree in earthworms. Data are described as mean±SD (n=3). Statistical significance compared with controls: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

3.4 Real-time PCR Analysis of Dinotefuran and Its Enantiomers

As is shown in Figure 3A, the relative expression of SOD gene was significantly up-regulated on the 7th and 14th day ($P<0.001$). After 28 days treatments, the expression levels of the SOD gene was more significantly decreased by the treatment of the high concentration of Rac-(±)-Dinotefuran and S-(+)-Dinotefuran groups (1.0 mg/kg and 0.5 mg/kg) than other treatment groups. The significant changes of the SOD gene showed that dinotefuran and its enantiomers caused toxic effects on earthworms and had a positive correlation with the exposure time and the dose.

The expression of MT gene is modulated by environmental stress, so it has been used to assess the eco-toxicity of contaminants (Fisker, 2016). As is shown in Figure 3B, after exposure for 7 days, a significant up-regulation trend was observed and the expression levels increased with an increase in the dose and exposure time ($P<0.001$). However, on the 14th day, the up-regulation trend in high concentration of S-(+)-Dinotefuran (1 mg/kg and 0.5 mg/kg) and 1mg/kg Rac-(±)-Dinotefuran, treatment groups disappeared. Until being exposured for 28 days, the expression levels of the MT gene down-regulated significantly on 1 mg/kg and 0.5 mg/kg treatments. Especially on the 28th day, S-(+)-Dinotefuran (1 mg/kg and 0.1 mg/kg), R-(-)-Dinotefuran (1 mg/kg) and Rac-(±)-Dinotefuran (1 mg/kg and 0.5 mg/kg) treatment groups were markedly lower than other treatment groups.

Heat shock protein 70 (HSP70) play an essential role in protecting cells from damages induced by environment pressure (Wang, 2015). As is shown in Figure 3C, after exposure for 3 days, a significant increase of expression of HSP70 gene was observed in the dinotefuran and its enantiomers treatment groups ($P<0.001$). After 14 days treatments, the relative expression levels of the HSP70 gene began to wane. On the 28th day, the relative expression levels of the HSP70 gene in all dinotefuran and its enantiomers treatment groups were starkly lower than the control group ($P<0.001$). The significant changes in HSP70 expression indicated that dinotefuran and its enantiomers caused stress in earthworms.

TCTP plays an important role in preventing cell apoptosis and causing tumor reversion (Wang, 2015). As is shown in Figure 3D, a significant up-regulation of the TCTP gene was observed in the presence of S-(+)-Dinotefur, R-(-)-Dinotefuran (1 mg/kg) and Rac-(±)-Dinotefuran (1 mg/kg and 0.5 mg/kg) on the days of 7 and 14 ($P<0.001$). On the days of 21 and 28, the relative expression levels of the TCTP gene were markedly lower than the control group except 0.1 mg/kg Rac-(±)-Dinotefuran treatments ($P<0.001$). The up-regulation at first and the following down-expression indicated that dinotefuran and its enantiomers may influence cell growth and lead to cell apoptosis at the final stage of the exposure.

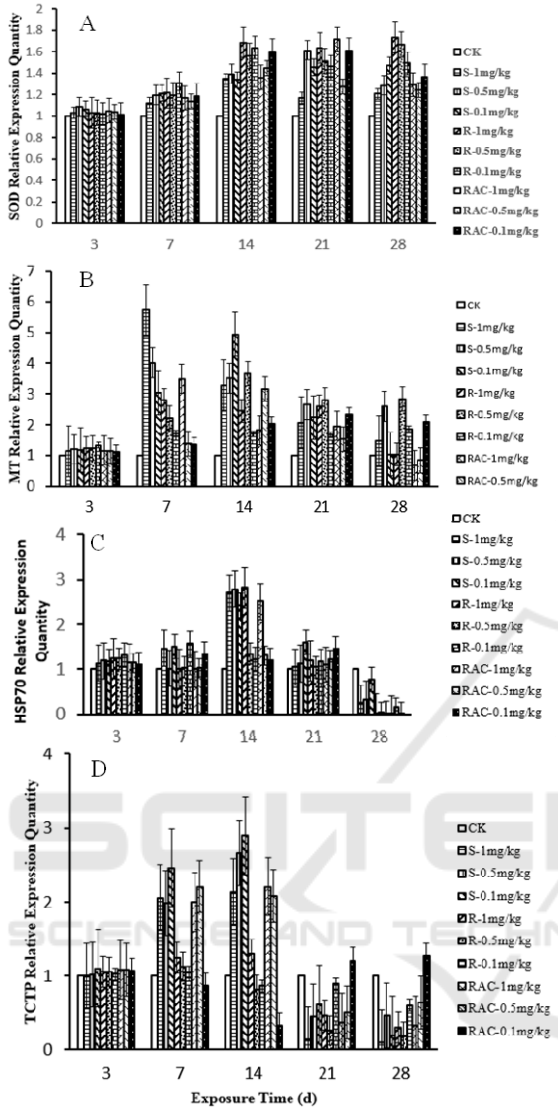

Figure 3: The effect of dinotefuran and its enantiomers(S-(+)-Dinotefuran and R-(-)-Dinotefuran) on the relative expression quantity of SOD (A), MT (B), HSP70 (C) and TCTP (D). Data are described as mean±SD (n=3). Statistical significance compared with controls: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

4 CONCLUSIONS

In this study, the biochemical and genetic toxicity of dinotefuran and its enantiomers on *Eisenia foetida* were evaluated from the individual, tissue, cell and molecule levels. The results showed that, dinotefuran and its enantiomers are moderately toxic to *Eisenia foetida* and have negative impacts on the earthworm at different levels. Both the exposure dose and time had obvious impacts on the toxicity. Moreover, increasing dose and time of dinotefuran and its enantiomers could induce redundant production of ROS, resulting in significant changes in antioxidant enzyme activities, DNA damage and the relative expression of functional genes.

This study explored the toxicity mechanisms underlying the toxicity of dinotefuran and its enantiomers on earthworms, and provided scientific basis references for devising and developing new environment-friendly pesticides.

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