Research Article

The Effects of Carbendazim on Acute Toxicity, Development, and Reproduction in *Caenorhabditis elegans*

Jie Li ¹, Xinghua Zhou ¹, Caiqin Zhang,¹ Yansheng Zhao,¹ Ying Zhu,¹ Jiayan Zhang,¹ Juan Bai,¹ and Xiang Xiao ^{1,2}

¹School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, China

²Jiangsu Jiangnan Biotech Co., Ltd., Zhanjiang 212300, China

Correspondence should be addressed to Xinghua Zhou; zxh2012@ujs.edu.cn

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Carbendazim, as a fungicide, was commonly used to control fungal diseases in agriculture, forestry, and veterinary medicines. In this study, the acute and reproductive toxicity of carbendazim was assessed using *Caenorhabditis elegans* (*C. elegans*) as a model in order to preliminarily evaluate the potential risks of this fungicide in agricultural production and application. The results showed that the growth of *C. elegans* was inhibited by 0.01 $\mu\text{g/L}$ carbendazim. The treatment of 0.1 $\mu\text{g/L}$ carbendazim caused a significant decrease in locomotion behavior and significant damage to the reproductive and antioxidant system, causing the lifespan of nematodes to be drastically shortened. These results provide a better understanding of the environmental risk of carbendazim and raise new concerns about safety.

1. Introduction

Pesticides, a kind of chemical or biological reagents, are widely used in agriculture to regulate plant growth and control diseases and insect pests, which can promote crop growth and improve crop yield [1]. However, the widespread use of pesticides will lead to different degrees of residues in crops or food and thus affect human health [2]. The problems of pesticide residues have not only attracted great attention of consumers but also become one of the key factors affecting food safety [3].

Carbendazim, as a broad-spectrum fungicide, has been used to control fungal diseases in agriculture, forestry, and veterinary medicines [4]. However, carbendazim is categorized in the hazardous category of chemicals by the World Health Organization [5] and has been classified in the priority list of endocrine-disrupting chemicals by the European Commission [6]. In recent years, it is obvious that the widespread use of carbendazim with over-range and over-dose and the fact that carbendazim is hard to be degraded both lead to the problem of carbendazim residues in

agriculture [7]. Although the toxic effect of carbendazim has been reported since the 1980s, the toxicity of carbendazim becomes a hot topic because of the increasing concern about environmental endocrine disruptors [4]. Carbendazim has been banned in several countries because of its negative impacts on the environment and health such as development and reproductive disturbances, toxicity, and mutagenicity [8]. The adverse effects of carbendazim on the biochemical, histopathological, and hematological parameters in the liver, kidney, and endocrine glands and their hormonal levels have been illustrated in rats [4]. Additionally, it needs further study on low concentration due to the residues of carbendazim.

Caenorhabditis elegans (*C. elegans*), as an important research model, is widely used to do some assessment. According to Amrit et al. [9], *C. elegans* has many advantages, such as small size, rapid generation time, easy of culturing on laboratory, and short adult lifespan. *C. elegans* was chosen in this study as the model organism to evaluate the toxicity of low concentration of carbendazim, which may be considered as a reference value for the application of carbendazim in agriculture.

2. Materials and Methods

2.1. Chemicals and Strains. Carbendazim (purity $\geq 99\%$; Aladdin® Biochemical Technology Co., LTD, Shanghai, China) was dissolved in N, N-dimethylformamide (DMF; Sinopharm Chemical Reagent Co., LTD, Shanghai, China) to produce 1 g/L carbendazim original solution. The concentrations of DMF were 0.1% in final exposure solutions (0.01, 0.1, 1, 10, 100 $\mu\text{g/L}$). 0.1% DMF without carbendazim was the control group. *C. elegans* (wild-type N2) were originally obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, MN, USA). The nematodes were cultivated on nematode growth medium (NGM) plates that were seeded with *Escherichia coli* OP50 at 20°C as described [10]. L1-larval *C. elegans* were collected by washing the gravid nematodes with a bleaching mixture (1 M NaOH, 10% NaHOCl).

2.2. Lethality. Carbendazim original solutions (1 g/L) were diluted with S liquid medium (1.12 g K_2HPO_4 , 5.92 g KH_2PO_4 , and 5.85 g NaCl were diluted with 1 L water) to get the final carbendazim concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mg/L, which contained 0.1% DMF. S liquid medium with 0.1% DMF was the control group. 30 nematodes (L4) were tested in 96-well plates for each concentration. The survival of nematodes was counted under a microscope after culturing for 24 hours in the incubator. The process of the test is based on the method of Xiang et al. [11]. Three parallel experiments were needed.

2.3. Locomotion Behavior. At least 10 *C. elegans* (L4) were picked randomly from each concentration to determine the locomotive behavior, which was recorded by both the head-thrash frequency and the body bend times [12]. The number of head-thrash frequency was counted by changes in the direction of bending at the midbody of *C. elegans* in 1 min. Measurement of body bend was defined as the times of the direction changes of the part of the nematodes cultured in NGM without *E. coli* OP50 in the 30 s.

2.4. Growth and Development Assays. *C. elegans* exposed to carbendazim for 24 h was analyzed. The body length of nematodes exposed to carbendazim was assessed by the Image J software. The offspring of each *C. elegans* from L4 larvae to day 1 was recorded at the L3 stage after individually transferring to a new plate every day until reproduction ceased [13]. At least three parallel tests were carried out.

2.5. Lifespan Analysis. All of *C. elegans* tested for lifespan were cultured in the same condition at 20°C. The synchronized *C. elegans* were cultivated in NGM plates with different concentrations of carbendazim until day 4. The tested nematodes then would be transferred into new NGM plates every 2 days. Surviving and dead *C. elegans* were recorded daily (beginning on the first day of adulthood) until all nematodes for each concentration had died [11]. At least three parallel tests were carried out.

2.6. Determination of Oxidative Damage. Intracellular ROS was measured with 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{DCFH-DA}$), which is the most common and sensitive reactive oxygen detection probe by far. The wild-type N2 *C. elegans* were washed in M9 buffer and then ultrasonically disrupted. The supernatant was analyzed the ROS level following the instruction of the ROS kit. The final working concentration of $\text{H}_2\text{DCHE-DA}$ was 10 μM [11]. The excitation and emission absorbance wavelengths were 485 nm and 535 nm, respectively. At least three parallel tests were carried out.

Intracellular total superoxide dismutase (T-SOD) was determined according to the instruction of the T-SOD kit purchased from the Nanjing Jiancheng Bioengineering Institute. After washed with M9 three times, the examined nematodes were ultrasonically disrupted and reacted with a T-SOD kit. The absorbance wavelength was 550 nm. In addition, the supernatant was used to detect the level of protein for each concentration, in which absorbance wavelength was 595 nm. At least three parallel tests were carried out.

2.7. Data Analysis. All data were given as mean $\pm$ standard error of the mean (SEM) by using one-way ANOVA. Graphs were presented using Origin 8.5 and GraphPad Primer 7, and statistical analysis was performed using the SPSS 19.0 software. The statistical significance level was conducted using * $p < 0.05$ and ** $p < 0.01$.

3. Results

3.1. Determination of the Locomotion Behavior of *C. elegans* after Carbendazim Acute Exposures. LC_{50} *C. elegans* were exposed to carbendazim for 24 hours to assess its acute toxic effects. Data are represented as shown in Table 1, and the obtained linear fitting equation was $y = 2.180x - 0.223$ through data analysis. The obtained LC_{50} is 0.867 mg/L.

Next, we assayed the determination of the locomotive behavior of *C. elegans* after carbendazim acute exposures by analyzing the data about head-thrash frequency and body-bending times of nematodes (Figures 1(a) and 1(b)). Both of them showed significant decreases at the carbendazim concentrations ranging from 0.01 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$ ($p < 0.01$). Additionally, the head thrashes of nematodes exposed to 100 $\mu\text{g/L}$ decreased to 68.27%. For the body bends test, when the carbendazim concentrations were 10 $\mu\text{g/L}$ and 100 $\mu\text{g/L}$, it had a significant inhibitory effect on the body bends of *C. elegans* by 36.77% and 35.48% compared with the control one, respectively.

3.2. Determination of the Growth and Development of *C. elegans* after Carbendazim Acute Exposures. Compared with the control group (Figures 1(c) and 1(d)), body length and body surface area were significantly ($p < 0.01$) reduced in the exposure groups from 0.01 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$. Both of them were decreased by 19.16% and 22.15% at the treatment of 0.01 $\mu\text{g/L}$ compared with the control group, respectively. The concentration of 10 $\mu\text{g/L}$ presented the most negative

TABLE 1: Effects of carbendazim on LC₅₀ *C. elegans* by 24-h acute exposures.

Concentration (mg/L)	Total	Survival
0	30	28
0.2	30	25
0.4	30	21
0.6	30	18
0.8	30	16
1.0	30	15

FIGURE 1: Effects of *C. elegans* on physiological traits exposed to carbendazim. (a) The head thrashes of *C. elegans* after carbendazim exposure; (b) the body bends of *C. elegans* after carbendazim exposure; (c) the body length of *C. elegans* after carbendazim exposure; and (d) the body surface area of *C. elegans* after carbendazim exposure. Data (mean $\pm$ SEM) are shown in figures as the percentage values compared with the control group. The asterisks present the significances between the each exposure group and control group (* $p < 0.05$ and ** $p < 0.01$).

impacts, and body length and body surface area of *C. elegans* were decreased by 35.21% and 65.22% compared with the control group, respectively.

3.3. Determination of the Brood Sizes of *C. elegans* after Carbendazim Acute Exposures. According to Figure 2, brood sizes of nematodes had a significant decrease ($p < 0.01$) in the treatment groups from 0.1 µg/L to 100 µg/L. The brood sizes of *C. elegans* decreased most significantly,

which decreased to 43.71% with the treatment of 10 µg/L compared with the control group.

3.4. Determination of the Lifespan of *C. elegans* after Carbendazim Acute Exposures. *C. elegans* lifespan was significantly inhibited by 0.01 µg/L to 100 µg/L carbendazim according to the lifespan curve shown in Figure 3. The results presented that *C. elegans* lifespan was decreased from 24 to 20 days with the treatment of 0.01 µg/L carbendazim. The

FIGURE 2: Effects of *C. elegans* on brood size exposed to carbendazim. Data (mean $\pm$ SEM) are shown as the percentage value compared with the control group. The asterisks present the significance between the each exposure group and control group (* $p < 0.05$ and ** $p < 0.01$).

FIGURE 3: Effects of *C. elegans* on survival exposed to carbendazim. Data (mean $\pm$ SEM) are shown as the percentage value compared with the control group. The asterisks present the significance between the each exposure group and control group (* $p < 0.05$ and ** $p < 0.01$).

lifespan of nematodes treated with 0.01 $\mu\text{g/L}$ carbendazim was reduced by 20.00%. When the carbendazim exposure concentration was 100 $\mu\text{g/L}$, the lifespan of *C. elegans* was reduced the most by 45.83%.

3.5. Effects of Acute Exposure to Carbendazim on the Anti-oxidant System of *C. elegans*. The ROS levels of control and treated *C. elegans* at carbendazim exposure of different concentrations are shown in Figure 4(a). It was indicated that the level of intracellular ROS was significantly increased ($p < 0.01$) at ranges from 0.01 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$ carbendazim. Compared with the control one, the ROS level was increased

at most by 70.60% with the treatment of 10 $\mu\text{g/L}$. According to the results of intracellular SOD levels, it had an increase at the treatment from 0.01 $\mu\text{g/L}$ to 100 $\mu\text{g/L}$ carbendazim (Figure 4(b)). The SOD level was increased by 10.70% at 0.1 $\mu\text{g/L}$ carbendazim compared with the control group.

4. Discussion

Carbendazim, as the fungicides, is widely used in agriculture to inhibit the growth of fungus. Carbendazim has been prohibited to use in Australia, most of the European Union, and the USA because of its severe toxicology and persistent nature [14]. For this study, it was the first time to use *C. elegans* as the model organisms to evaluate the effects of carbendazim on locomotive behavior, growth and development, reproduction, lifespan, and antioxidant systems. Moreover, results showed that it had a negative influence on *C. elegans*.

According to the 24 h-LC₅₀, the acute toxicity concentration of *C. elegans* exposed to different concentrations of carbendazim is 0.867 mg/L. The 96-h LC₅₀ of carbendazim in response to zebrafish has been illustrated as 1.75 mg/L [15]. The eggs of Prussian carp *Carassius gibelio* has shown the toxicity effects at the concentration of 0.036 mg/L [16, 17]. Studies presented that the growth and development of *Navicula* sp. is inhibited by carbendazim with a 24 h-EC₅₀ value of 2.18 mg/L. Though the rate of algal growth is recovered after 72-h exposure, the chlorophyll-a content remains significantly decreased when the treatment of carbendazim was beyond 0.5 mg/L [18]. In this present, *C. elegans* exposed to a low concentration of carbendazim were selected to evaluate its effects depending on the actual concentration of human daily exposure. Low concentrations of carbendazim do not mean it is safe. Carbendazim shows negative biological impacts at much lower doses in some studies.

Locomotive behavior was evaluated to assess the neurotoxicity of *C. elegans* (L4 larva) after 24-h exposure to carbendazim. The results showed that carbendazim could have negative effects on locomotive behavior through the detection of head thrashing and body bending of *C. elegans*, which both were more sensitive at the higher exposure group. The locomotive behavior of zebrafish embryos exposed to carbendazim is sensitive [15]. Previous studies have shown that fish have an abnormal behavior when sublethal concentrations of carbendazim are 0.22–0.43 mg/L [19].

Developmental malformations could also be one reason for abnormal locomotion [20]. The growth and development of *C. elegans* were assayed in our study. Results showed that the body length and body area of *C. elegans* were significantly narrowed at the treatment exceeding 0.01 $\mu\text{g/L}$ carbendazim. The normal growth of vertebrates is related to the metabolic thyroid hormone homeostasis [21, 22]. Williams et al. [23] have indicated that carbendazim could cause sperm loss after implantation, fetal malformation, and slow growth and development.

The reproductive toxicity of carbendazim has been demonstrated that carbendazim could inhibit the microtubule polymerization of fungal and mammalian cells, causing disruption of microtubule assembly by acting with

FIGURE 4: Effects of *C. elegans* on antioxidative system exposed to carbendazim. (a) The ROS level of *C. elegans* after carbendazim exposure; (b) the SOD level of *C. elegans* after carbendazim exposure. Data (mean $\pm$ SEM) are shown as the percentage value compared with the control group. The asterisks present significance between the each exposure group and control group (* $p < 0.05$ and ** $p < 0.01$).

β -tubulin, which results in impairing the segregation of chromosomes in the process of cell division [24]. The formation of microtubules by noncovalent bindings of α - and β -tubulin is responsible for chromosome segregation in the process of mitosis and meiosis [24]. The brood size of *C. elegans* significantly decreases at 0.1 µg/L carbendazim concentration. Carbendazim has been found to affect the reproduction systems in Japanese quails [25] and hamsters [23]. It was concluded that *C. elegans* lifespan was significantly decreased with carbendazim concentration of ≥ 0.01 µg/L based on our study. Studies have shown that carbendazim has led to infertility and developmental toxicity and manifests embryo toxicity, germ cell apoptosis, and teratogenesis in different mammalian species [17, 18, 24].

Apoptosis is a complex programmed cell death, which is a highly regulated phenomenon characterized by a series of cellular processes [26, 27]. Many studies have presented that the production of ROS induced by oxidative stress is related to apoptotic cell death [28]. Our study found that carbendazim could induce a significant increase in the level of ROS values and a little increase in the level of SOD values. Oxidative stress caused by environmental pollution induces the increased expression of ROS and subsequently damages the antioxidant defense system [29]. SOD is responsible for the detoxification of toxic free radicals and their activities, which is used to evaluate the oxidative stress level and cellular antioxidant status [17, 24]. Metalloenzyme SOD accelerates the transformation of endogenous cytotoxic superoxide radicals to H_2O_2 , and the increase of SOD expression levels may contribute to improving the enzyme activities in order to eliminate the superoxide radicals induced by carbendazim and to prevent the occurrence of cellular dysfunction during exposure of carbendazim [24, 29]. Higher exposure concentrations of carbendazim could cause severe oxidative stress, which subsequently destroys the balance of cell homeostasis and promote apoptosis [30]. However, carbendazim in low concentrations could still significantly damage the reproductive system according to our results.

5. Conclusion

As far as we know, the present study evaluated the safety of carbendazim exposed to *C. elegans* for the first time. It demonstrated that carbendazim could have a harmful effect on the locomotive behavior, development and growth, reproduction, lifespan, and antioxidant system of *C. elegans*. Hope that it needs to pay more attention to the application of carbendazim based on the results. In addition, the safety of carbendazim for use needs to evaluate further, especially the bioaccumulation toxicity and potential genotoxic effects.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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