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Reproductive toxicity of enrofloxacin in *Caenorhabditis elegans* involves oxidative stress-induced cell apoptosis

Jiahao Huang[1], *Lizi Liao*[2], *Guowei Wang*[2,3,*], *Zhongkun Du*[4], *Zhengxing Wu*[1]

[1] *Key Laboratory of Molecular Biophysics, Ministry of Education, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China*

[2] *School of Environmental Ecology and Biological Engineering, Wuhan Institute of Technology, Wuhan 430205, China*

[3] *Key Laboratory of Green Chemical Engineering Process of Ministry of Education, Wuhan 430205, China*

[4] *College of Resources and Environment, Shandong Agricultural University, Taian 271018, China*

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ABSTRACT

Fluoroquinolone antibiotics (FQs) that persist and bioaccumulate in the environment have aroused people's great concern. Here, we studied the adverse effects of FQs in soil animals of *Caenorhabditis elegans* via food-chronically exposure. The result shows *C. elegans* exposed to FQs exhibited reproductive toxicity with small-brood size and low-egg hatchability. To study the underlying mechanism, we conduct a deep investigation of enrofloxacin (ENR), one of the most frequently detected FQs, on nematodes which is one of commonly used animal indicator of soil sustainability. The concentration-effect curves simulated by the Hill model showed that the half effect concentrations (EC_{50}) of ENR were (494.3 ± 272.9) µmol/kg and (107.4 ± 30.9) µmol/kg for the brood size and the hatchability, respectively. Differential gene expression between the control and the ENR-exposure group enriched with the oxidative stress and cell apoptosis pathways. The results together with the enzyme activity in oxidative stress and the cell corpses suggested that ENR-induced reproductive toxicity was related to germ cell apoptosis under oxidative stress. The risk quotients of some soil and livestock samples were calculated based on the threshold value of EC_{10} for the egg hatchability (2.65 µmol/kg). The results indicated that there was possible reproductive toxicity on the nematodes in certain agricultural soils for the FQs. This study suggested that chronic exposure to FQs at certain levels in environment would induce reproductive toxicity to the nematodes and might reduce the soil sustainability, alarming the environment risks of antibiotics abuse.

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Introduction

Antibiotics as the drugs against bacteria have been used in curing diseases and promoting the growth of livestock animals for several decades (Sarmah et al., 2006; Van Boeckel

* Corresponding author.
E-mail: gwwang@wit.edu.cn (G. Wang).

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et al., 2014). Fluoroquinolone antibiotics (FQs) are presently one of the most popular classes of antibiotics that have been used worldwide in human health and animal breeding due to their strong antibacterial activity, broad antibacterial spectrum, and low cross-resistance (Riaz et al., 2017; Van Boeckel et al., 2014; Tomas et al., 2019). New researches recently showed that FQs could inhibit the frameshifting efficiency of the COVID-19 and other coronaviruses (Bhatt et al., 2021; Sun et al., 2021). In fact, those antibiotics were not completely absorbed or metabolized by humans and animals. A substantial proportion (30%-90%) of antibiotics was excreted with the parent form and/or metabolites (Hanna et al., 2018), and accumulated into the environment owing to their long-term persistence (Liu et al., 2017). FQs have been frequently detected at high concentrations in soil and water environment associated with agriculture (Riaz et al., 2018; Zhang et al., 2015; Zhu et al., 2013). It was reported that livestock manures possessed a high concentration of FQs in the range from 14.7 µmol/kg to 3951 µmol/kg (Karcı and Balcıoğlu, 2009; Martínez-Carballo et al., 2007; Zhao et al., 2010). Since the livestock manures were directly or indirectly spread into farmland as fertilizers, FQs have been frequently detected in manure-fertilized agricultural soils at the mg/kg level (Li et al., 2021). Most soil-dwelling organisms suffered from the FQs (Zhou et al., 2020).

The adverse effects of FQs were investigated for evaluation of the ecological risks of FQs that were spread into the environment. Several studies have demonstrated that FQs could disturb the development and growth of some organisms including zebrafish, duckweed, and fruit fly (Gomes et al., 2017; Liu et al., 2019a; Xi et al., 2019). However, there were indeed limited studies on the toxicity of FQs to the soil animals despite the frequent detection of FQs in soils. Li et al. (2016) reported that the burrowing activity and CO_2 production of the earthworm *Eisenia fetida* were significantly reduced after its exposure to enrofloxacin (ENR) at the concentration of 27.8 µmol/kg. The adverse effects of norfloxacin (NOR) on the soil collembolan *Folsomia candida* were to decrease the population of the predominant bacteria in the gut and finally inhibit the collembolan's normal growth (Zhu et al., 2018). Overall, there are limited studies on the toxicity of FQs to soil animals, making it difficult to accurately assess the ecological risk of FQ in soils.

Soil nematodes are one of the most abundant and diverse groups of soil biota in terrestrial ecosystems (Schratzberger et al., 2019). They are important regulators of soil microbial productivity, nutrient cycling and plant growth (Bastow, 2020; Zhang et al., 2019), and are considered excellent indicators of soil sustainability (Mills et al., 2020). However, up to date, the toxicity of FQs on the soil nematodes is rarely known. Herein, the toxic effects of FQs on the soil nematodes were investigated using the model nematode of *Caenorhabditis elegans*. The growth and fecundity of the *C. elegans* were measured after exposure to FQs. The reproductive toxicity of FQs on the nematode was observed and the underlying mechanism of the toxic effects was explored by analysis of the transcriptional level of the whole transcriptome, the activity of the antioxidant enzyme involved in oxidative stress, and the cell apoptosis in *C. elegans*. This study provides important information for assessing the ecological risk of FQs in soils.

1. Materials and methods

1.1. *Chemicals, nematode strain, and culture*

The FQ antibiotics, including enrofloxacin (ENR, CAS: 93106-60-6), ciprofloxacin (CIP, CAS: 85721-33-1), norfloxacin (NOR, CAS: 70458-96-7), ofloxacin (OFL, CAS: 82419-36-1), fleroxacin (FLE, CAS: 79660-72-3), lomefloxacin hydrochloride (LOM, CAS: 98079-52-8), and sarafloxacin hydrochloride (SAR, CAS: 91296-87-6) with all of the purities > 98%, were purchased from J&K Chemical (Beijing, China). Molecular structures of the seven FQs were shown in **Appendix A Fig. S1**. The superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GSH-PX) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). PrimeScript^{TM} RT-PCR and SYBR® Premix Ex Taq^{TM} (Tli RNaseH Plus) kits were purchased from Tiangen Biochemical Technology Co., Ltd. (Beijing, China). The sources of the other chemicals, including acridine orange (AO), levamisole, Triton X-100, and the two antioxidants of N-acetyl-L-cysteine (NAC) and β-carotene, are provided in the **Appendix A Text S1.1**.

Nematode strain Bristol N2 was acquired from the Caenorhabditis Genetics Center (http://www.cbs.umn.edu/CGC/). The worm strain was cultured in Petri dishes (inner diameter 90 mm) on nematode growth medium (NGM) seeded with *Escherichia coli* OP50 at (21±1)°C in accordance with the standard protocol (Brenner, 1974).

1.2. *Worm exposure*

Firstly, the Petri dishes for the experiment were prepared as the procedure provided in the **Appendix A Text S1.2**. The final concentrations of individual FQ in the Petri dishes are set to 0, 5.56, 13.9, 27.8, 55.6, 139, 209, 278, and 556 µmol/kg. Then, 50 of the synchronized L1 *C. elegans* were transferred into each of the Petri dishes. The dishes were sealed with parafilm and placed in a backlit place at 20°C. The nematodes were exposed for 48 hr and then collected for various experiments.

1.3. *Growth and reproduction monitoring*

The growth rate was determined by counting the number of nematodes at every developmental stage after 48 hr of cultivation as previously reported (Shen et al., 2018). The pharyngeal pumping rate of worms was measured directly by counting the number of pharyngeal contractions of 10 randomly-picked out worms in each treatment at the adult day 1 stage (Liu et al., 2019b).

Reproduction was monitored through the endpoints of brood size and egg hatchability of the *C. elegans* (Du et al., 2015; Zhao et al., 2016a). After 48 hr exposure, the nematodes were individually picked out to a new NGM plate with fresh food every 24 hr, and allowed to lay eggs until the end of the reproductive period. The total number of eggs laid per nematode is defined as brood size. Eggs from the reproductive period were incubated at 20°C and monitored under the Zeiss Discovery V8 stereomicroscope coupled with the CCD camera. The number

of hatched-out offspring from individual worms was counted when the offspring were grown to L3 and/or L4 stage. Then, hatchability was determined as the ratio of the hatched number over the brood size.

1.4. *Morphology determination of eggs in the uterus*

To monitor eggs in the uterus, we transferred the adult hermaphrodites in the tested and control groups individually to a drop of levamisole solution (25 μmol/L) on a glass slide covered by an agar pad (2%, W/V). Morphology of the eggs in the uterus was imaged under an Andor Revolution XD laser confocal microscope system (Yokogawa Electric Corporation, Tokyo, Japan) combined with an Olympus IX-71 inverted microscope (Olympus, Tokyo, Japan). The images were captured by the Andor iXon^{EM} + DV-885K EMCCD camera and analyzed using the ImageJ software (Liu et al., 2019b). For each treatment, there were six replicates.

1.5. *RNA extraction, RNA-Seq and qRT-PCR*

The synchronized L4 stage nematodes were collected and washed four times using M9 buffer solution (mixed solution of 86 mmol/L NaCl, 42 mmol/L Na_2HPO_4, 22 mmol/L KH_2PO_4, and 1 mmol/L $MgSO_4$) to clear away the bacteria. Then, the total RNA of the nematodes was isolated by Trizol reagent (Invitrogen) in accordance with the manufacturer's guidelines. The purity and concentration of the RNA were determined using the Nanodrop spectrophotometer. The cDNA synthesis was performed using the isolated nematode RNA (about 1 μg) and the cDNA Synthesis Kit with random primers. The cDNA fragments were ligated with Illumina sequencing adapters after the addition of poly (A) and end repair. Subsequently, the ligation products were enriched by PCR amplification to construct the cDNA library template. At last, the library was sequenced using Illumina HiSeqTM 2500 by Gene Denovo Biotechnology Co. (Guangzhou, China).

RNA-seq results were validated using quantitative real-time polymerase chain reaction (qRT-PCR). The relative transcriptional levels of the selected genes in the main text were measured under qRT-PCR with gene-specific primers (**Appendix A Table S1**).

All reactions were conducted in quadruplicate. The relative quantification was determined by the expression ratio of targeted genes over the reference β-actin gene, and the final results were expressed as the relative expression ratio normalized by z-score.

1.6. *Antioxidant activity and germline apoptosis evaluation*

Treatment plates were prepared in the same way as before and antioxidants were separately added into the NGM. Then dishes were sealed with parafilm and placed in a backlit place after dosing (including the blank group and only ENR-exposed group).Worms were exposed to the ENR (0 (the control), 139, 278 and 556 μmol/kg), NAC (6.17 μmol/kg), β-carotene (18.6 μmol/kg), ENR (139 μmol/kg) + NAC (6.17 μmol/kg), ENR (139 μmol/kg) + β-carotene (18.6 μmol/kg), ENR (278 μmol/kg) + NAC (6.17 μmol/kg), ENR (278 μmol/kg) + β-carotene, respectively, for 3 days as described above, and then, washed four times using M9 buffer solution and diluted to 1% (V/V). The activity of SOD, CAT, and GSH-PX was determined by the CAT, SOD, and GSH-PX assay kit, and the level of GSH was determined by the GSH assay kit following the manufacturer's instructions. Briefly, the synchronized L4 stage nematodes were collected and homogenized. Then, the supernatant of the centrifuged lysate was taken for the SOD, CAT and GSH-PX activity measurement and the GSH level detection. The level of GSH is expressed as micromoles per gram of protein while the activities of SOD, CAT and GSH-PX are expressed as unit per milligram of protein. For each treatment, six replicates were performed.

The germ cell apoptosis was analyzed using the AO staining method (Qu et al., 2019). To determine germ cell apoptosis, approximately 12 nematodes from each treatment were stained with 5 mg/L of AO solution for 10 min in darkness at room temperature. After washing with M9 buffer more than 3 times, the worms were observed under an Andor Revolution XD laser confocal microscope system. Then, Image J was used to measure the relative fluorescence intensity. Fifty animals were observed per treatment.

1.7. *Effect and environmental risk analysis*

Effect analyses for ENR reproductive toxicity were performed using the outcomes of brood size and egg hatchability in *C. elegans*. The concentration-effect curve for every outcome was obtained by fitting the Hill model (Eq. (1)):

$$E = \frac{E_{max} \times C^n}{EC_{50}^n + C^n} \quad (1)$$

where, E (%) is the decreased ratio of brood size or the hatchability of eggs in *C. elegans;* C (μmol/kg) is the exposure concentration of ENR; E_{max} is the calculated maximal effect; EC_{50} (μmol/kg) is the ENR concentration of a half-maximal effect; n is the coefficient implying the correlation of ENR to the outcomes.

The environmental risk of ENR in soil samples was evaluated by the risk quotient method (Cheng et al., 2018; Huang et al., 2021). Firstly, the threshold value of ENR reproductive toxicity causing a 10% effect (EC_{10}) was calculated by the Weibull model (Cheng et al., 2018). Next, the risk quotient (RQ) was obtained on the basis of the threshold value of EC_{10} (Eq. (2)):

$$RQ = \frac{C_s}{T} \quad (2)$$

where, RQ > 1 suggests that reproductive toxicity caused by ENR in a soil sample is possible; C_s (μmol/kg) is the ENR concentration in the soil sample. T is the threshold of EC_{10}. Detailed methods for the data statistical analysis have been provided in the **Appendix A Text S1.3**.

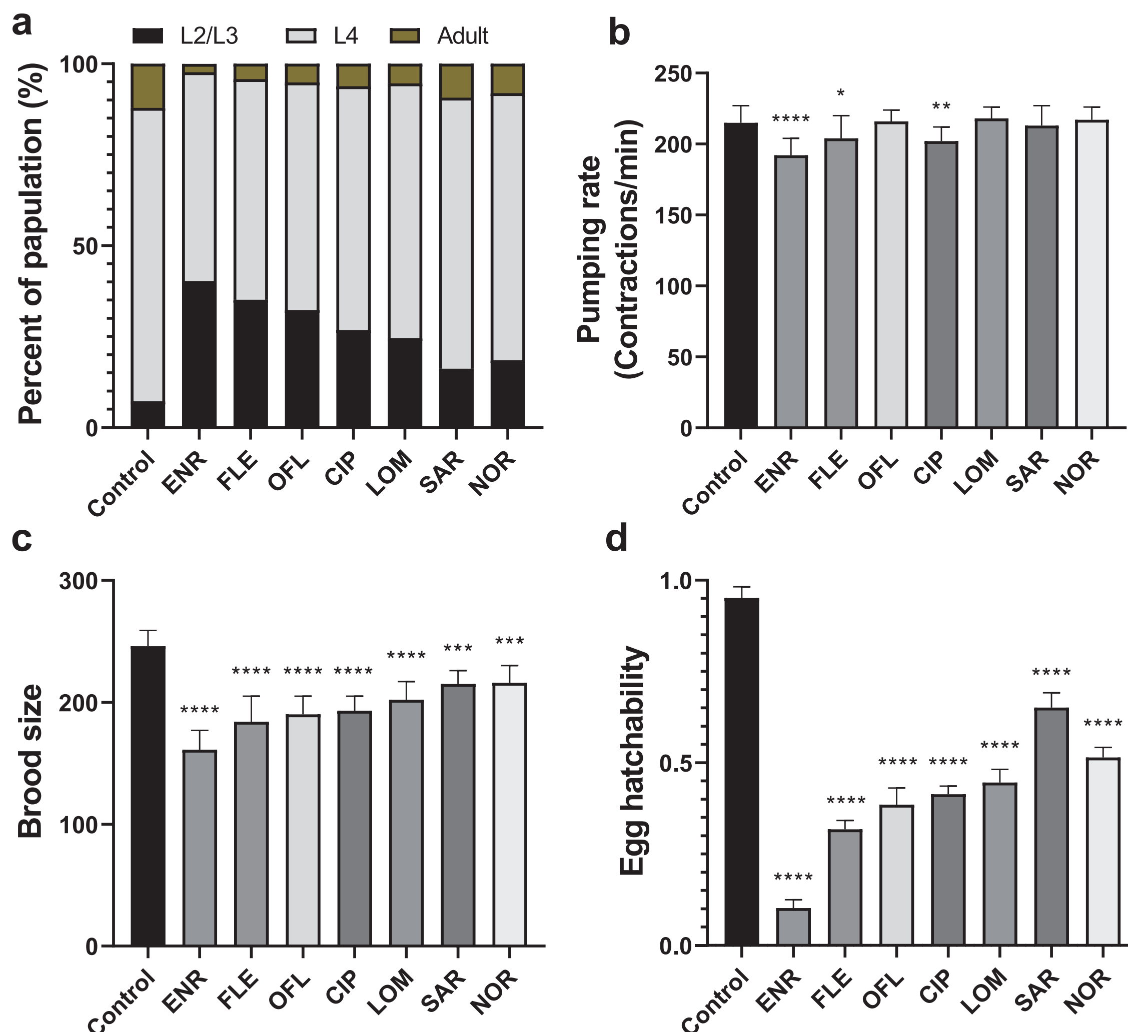

Fig. 1 – Effects of Fluoroquinolone antibiotics (FQs) on the growth rate, pharyngeal contraction and reproduction of *Caenorhabditis elegans*. (a) The percent of worms at each developmental stage after 48 hr of cultivation, (b) the pharyngeal pumping rate of the worms at the adult day 1 stage, (c) the brood size of worms and (d) the hatchability of the eggs. The test number of (a) was $n \geq 2$ plates and 50 worms/plate for each treatment, while the test number of (b-d) was $n \geq 10$ worms for each treatment. Statistical significance is indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ in comparison with value from the control (Student's *t*-test).

2. Results and discussion

2.1. *Physiologic toxicity of FQs exposure*

2.1.1. *Toxicity of FQs on growth rate and reproduction of* C. elegans

The growth rate and reproduction were examined at individual FQ concentrations of 278 µmol/kg (100.1, 92.3, 88.9, 102.8, 108.1, 117.5 and 100.6 mg/kg for ENR, CIP, NOR, FLE, LOM-HCl, SAR-HCl and OFL, respectively). As shown in Fig. 1a, 79%–84% of the nematodes were at L4 stage and 6%-8% were at L2/L3 stages in the control after 48 hr of incubation, whereas 57%-75% of the nematodes were at L4 stage and 16%-41% was at L2/L3 stages in the groups tested with individual FQ. Results indicated that the growth rate of *C. elegans* was suppressed after exposure to FQs. The food intake of *C. elegans* depends on the pharyngeal contraction. Several FQs such as ENR, FLE and CIP showed varying degrees of inhibition on the pharyngeal contraction, while other FQs including NOR, LOM-HCl, SAR-HCl and OFL had no significant effects on the pharyngeal contraction (Fig. 1b). Although the pharyngeal contraction of nematodes was partially inhibited by the ENR, FLE and CIP, it was still maintained at a high level (>180). Thus, the influence of food intake on the growth of nematodes was negligible.

The effect of FQs on the reproduction of *C. elegans* was monitored based on the brood size and hatchability. As shown in Fig. 1c and d, the brood size per nematode and the hatchability of the eggs were decreased significantly after exposure to the FQs. ENR exhibited the highest inhibition strength on the growth rate and reproduction among the seven FQs, showing the highest portion (37%-45%) of nematodes at L2/L3 stage, the lowest brood size per nematode, and the lowest hatchability for the eggs. Therefore, ENR was studied subsequently as a

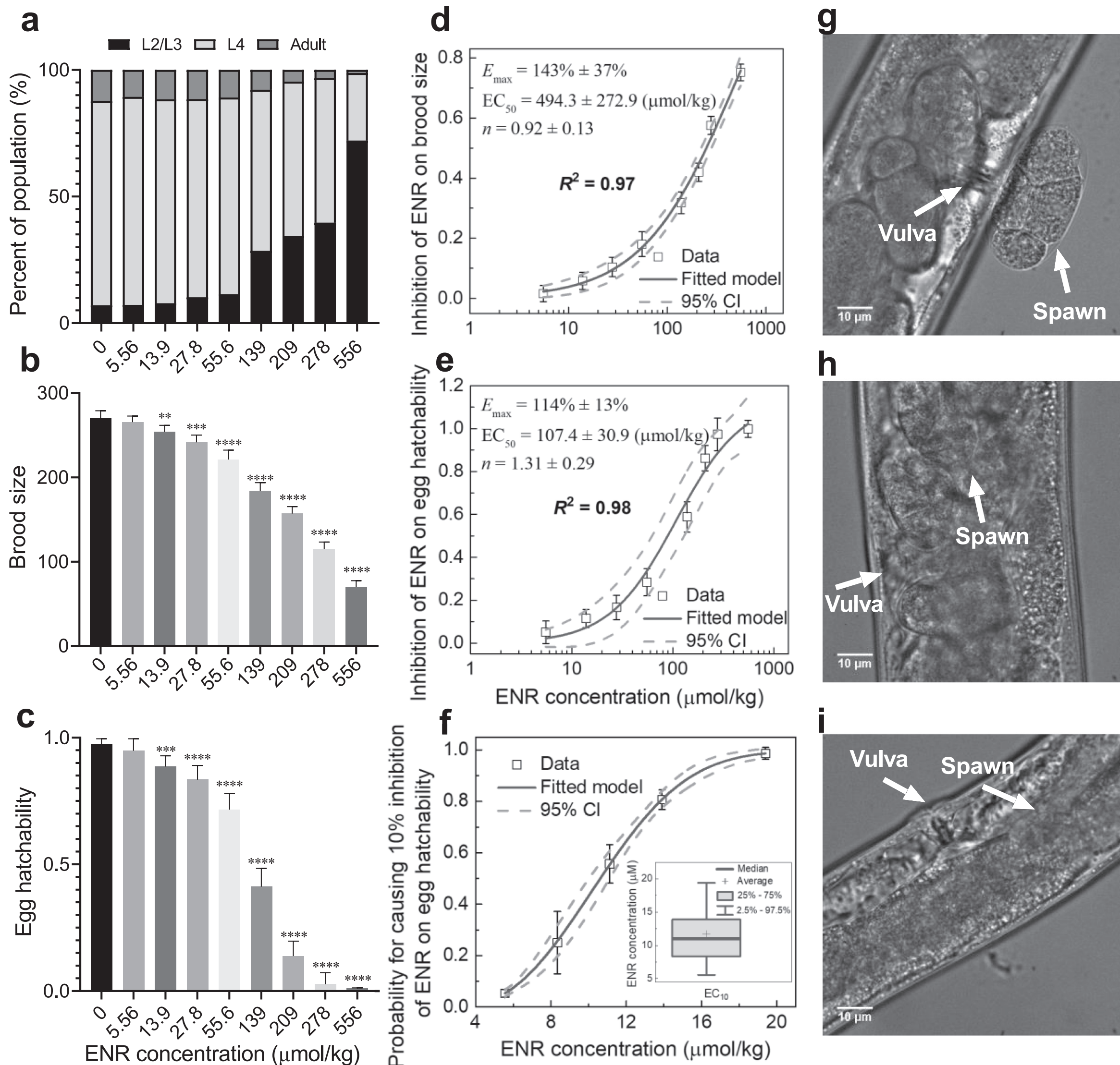

Fig. 2 – Effects of enrofloxacin (ENR) on the growth rate and reproduction of *C. elegans*. (a) The percent of worms at each developmental stage, (b) the brood size of worms and (c) the hatchability of the eggs in each treatment. Concentration-effect analysis of (d) the brood size or (e) egg hatchability associated with reproductive toxicity induced by ENR in *C. elegans*. (f) box and whisker plots of ENR concentrations corresponding to percentiles of data points extracted from 10% inhibition of eggs' hatchability. Shape of the eggs in the uterus of worms at the adult day 1 stage (g) in the control group and the groups treated with (h) 139 μmol/kg and (i) 556 μmol/kg ENR. The test number of (a) was $n \geq 2$ plates with 50 worms/plate and (b and c) was $n \geq 10$ worms for each treatment. **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ in comparison with the value for the control (one-way ANOVA followed by Dunnett's test).

representative to explore the inhibitory mechanism of FQs on the development and reproduction of *C. elegans*.

2.1.2. Toxicity of ENR on the growth rate and reproduction of C. elegans

The percent of nematodes at the stages of adult, L4 and L2/L3 for the exposure to no more than 27.8 μmol/kg ENR was similar to that of nematodes in the control (Fig. 2a). As the exposure concentration increased to 139-556 μmol/kg, the development of nematodes was frequently arrested in L2/L3 stages, demonstrating that exposure to ENR would suppress the development of *C. elegans*. The reproduction was reflected in the brood size of nematodes and the hatchability of eggs. When the *C. elegans* were exposed to 13.9-556 μmol/kg of ENR, the brood size of nematode and the hatchability of the eggs were significantly decreased (Fig. 2b and c). For example, when *C. elegans* grew to 3 days old, the nematodes in the control group laid a lot of eggs, but the nematodes exposed to 278 and 556 μmol/kg ENR of the same period laid little eggs (**Appendix A Fig. S2**).

The concentration-effect curves were generated based on the data from Fig. 2b and c using the Hill model to illustrate the relationship between reproductive toxicity and ENR (Fig. 2d and e). The E_{max} is 143% ± 37%; EC_{50} is (494.3 ± 272.9) μmol/kg,

and n is 0.92 ± 0.13 ($R^2 = 0.97$, $p < 0.001$) for the brood size, while the E_{max} is 114% ± 13%; EC_{50} is (107.4 ± 30.9) µmol/kg, and n is 1.31 ± 0.29 ($R^2 = 0.98$, $p < 0.001$) for the hatchability. A comparison of the brood size and hatchability revealed that hatchability was more sensitive to ENR exposure in *C. elegans*. Therefore, the threshold of EC_{10} for the inhibition of hatchability was applied to calculate the RQ values for environmental risk assessment. EC_{10} was estimated to be 11.12 (95% CI 5.56–19.4) µmol/kg (Fig. 2f). The Weibull threshold model simulated our data best and defined the ENR threshold value of EC_{10} for the hatchability of egg in C. *elegans* as (2.65 ± 0.24) µmol/kg.

2.1.3. *Shape variation of the eggs*

The morphology of eggs in the uterus was observed under confocal microscopy to investigate the reasons for the decrease in brood size and egg hatchability. As shown in Fig. 2g, eggs in the control were normal oval shape. However, some of the eggs from the nematodes treated with ENR were developmentally abnormal with the shape of flat and irregular ellipses (Fig. 2h and i). The abnormal development was also reflected in the laid eggs in the NGM plates (**Appendix A Fig. S3**). The eggs in the control with high hatchability were oval shape (**Appendix A Fig. S3a**) while some of the eggs from the ENR-exposed nematodes with low hatchability were rough, brown and lack of egg shell (**Appendix A Fig. S3b** and **c**). The results indicated that exposure to ENR would induce the abnormal development of eggs in the nematodes, resulting in weak hatchability.

2.2. *Mechanism exploration for the ENR induced reproductive toxicity*

2.2.1. *Effects of the ENR on genes expression*

To investigate the mechanism of ENR induced reproductive toxicity, transcriptional levels of the whole transcriptome in *C. elegans* have been analyzed. Certain genes of significantly differential expression were validated using the qRT-PCR. The exposure group at the concentration of 139 µmol/kg ENR has been chosen to be tested due to the variation of genes expression and the relatively low concentration. According to the results from the whole transcriptome and qRT-PCR, the exposure to ENR significantly ($p < 0.05$) decreased the transcriptional levels of *mev-1, sod-2, gst-4, gst-5* genes and increased the transcriptional levels of *sod-1, gst-1, gst-3, gst-8, gpx-2, gpx-3, ctl-1, ctl-2* genes in *C. elegans* (Fig. 3a). These genes have an essential role in mediating oxidative stress in nematodes (Yu et al., 2020). For example, the increased expression of the genes *ctl-1* and *ctl-2* indicates that a protection response has been induced (Qu et al., 2018), and the increased expression of genes *gst-1 gst-3* and *gst-8* suggests that parts of the inherent defense processes have been activated to help protect *C. elegans* from oxidative damage (Lu et al., 2020). On the other hand, the decreased expression of the gene *sod-2* suggests inhibition of the antioxidation defense system on nematodes (Qiu et al., 2020), while the decreased expression level of gene *mev-1* means an increase in the cellular titer of reactive oxygen species (ROS) which will lead to an increase of the oxidative stress (Rajaei et al., 2021). Further analysis demonstrated that most of the other genes in the same pathway as these genes were also differentially expressed (**Appendix A Fig. S4**). The results indicated that the oxidative stress response might have a key role in the ENR-induced reproductive toxicity in nematodes. Wu et al. (2011) have reported that the change of transcriptional levels of genes related to oxidative stress was closely associated with the decreased reproductive capacity of *C. elegans*. The *in vivo* researches have also shown that exposure to ENR and other FQs such as CIP induced the generation of ROS and caused toxic effects on the oxidative stress enzymes, resulting in oxidative damage (Gomes et al., 2017; Peltzer et al., 2017). ROS generation induced by wheat gluten hydrolysate (500 mg/L) causes germ cell apoptosis in *C. elegans* (Min et al., 2019). Paraquat (5 mmol/L) significantly increased ROS formation in nematodes and reduced the chemotaxis, the physiological lifespan, and the survival in assays for heat-stress resistance (Dilberger et al., 2019).

Cell apoptosis pathways were also observed from the whole transcriptome and validated by qRT-PCR. The exposure to ENR at a level of 139 µmol/kg significantly ($p < 0.05$) decreased the transcriptional levels of *egl-38, egl-5, egl-13, pax2-b, pax3, dpl-1, ced-11* genes and increased the transcriptional levels of *egl-3* and *ced-7* genes in C. *elegans* (Fig. 3 b). These genes themselves or mammalian homologs have an essential role in mediating germ cell apoptosis in nematodes (Hengartner, 1997; Polli et al., 2014). Further transcriptomic analysis revealed that the other gene expressions of the same pathways were differentially expressed (**Appendix A Fig. S5**), indicating that germ cell apoptosis should play an important role in the reproductive toxicity of ENR in nematodes. It has been reported that toxicants caused oxidative stress which resulted in cell apoptosis (Hunt et al., 2019; Krishnaswamy et al., 2010; Collins et al., 2008). Pathways associated with the regulation of oxidative responses and germ cell apoptosis are involved in several chemical-induced nematode reproductive toxicity (Wang et al., 2012, 2017; Zhao et al., 2016b). Therefore, the reproductive toxicity caused by ENR in nematodes has been hypothesized via the germ cell apoptosis under oxidative stress. Further investigations have been performed in the next section to determine the oxidative stress and cell apoptosis of nematodes after exposure to ENR.

2.2.2. *Effects of the ENR on antioxidant enzyme system*

Oxidative stress induces changes in the activity of oxidative enzymes and the content of the redox substances in biota (Stojiljković et al., 2005). In this study, the activity levels of SOD, CAT, GSH-PX and the content of GSH in *C. elegans* were decreased significantly with the increase of ENR (Fig. 4), indicating that ENR at the exposure concentrations induced apparent oxidative stress. It has a similar finding from the soil Collembola after exposure to another FQ of NOR (Li et al., 2021). The SOD, CAT, GSH-PX and GSH are the most important antioxidant enzymes or substances which play vital roles in maintaining the endogenous redox homeostasis (Li et al., 2017; Lushchak, 2012). All of them, together with other antioxidant enzymes or substances, constitute a steady-state antioxidant defense system to protect the organism against xenobiotic pollutants in the early stages (Li et al., 2017). In this study, the significant reduction of the activities of the antioxidant enzymes and substance indicated that the endogenous redox homeostasis in nematodes was damaged. The damage to the antioxidant defense system could result in the damage of cel-

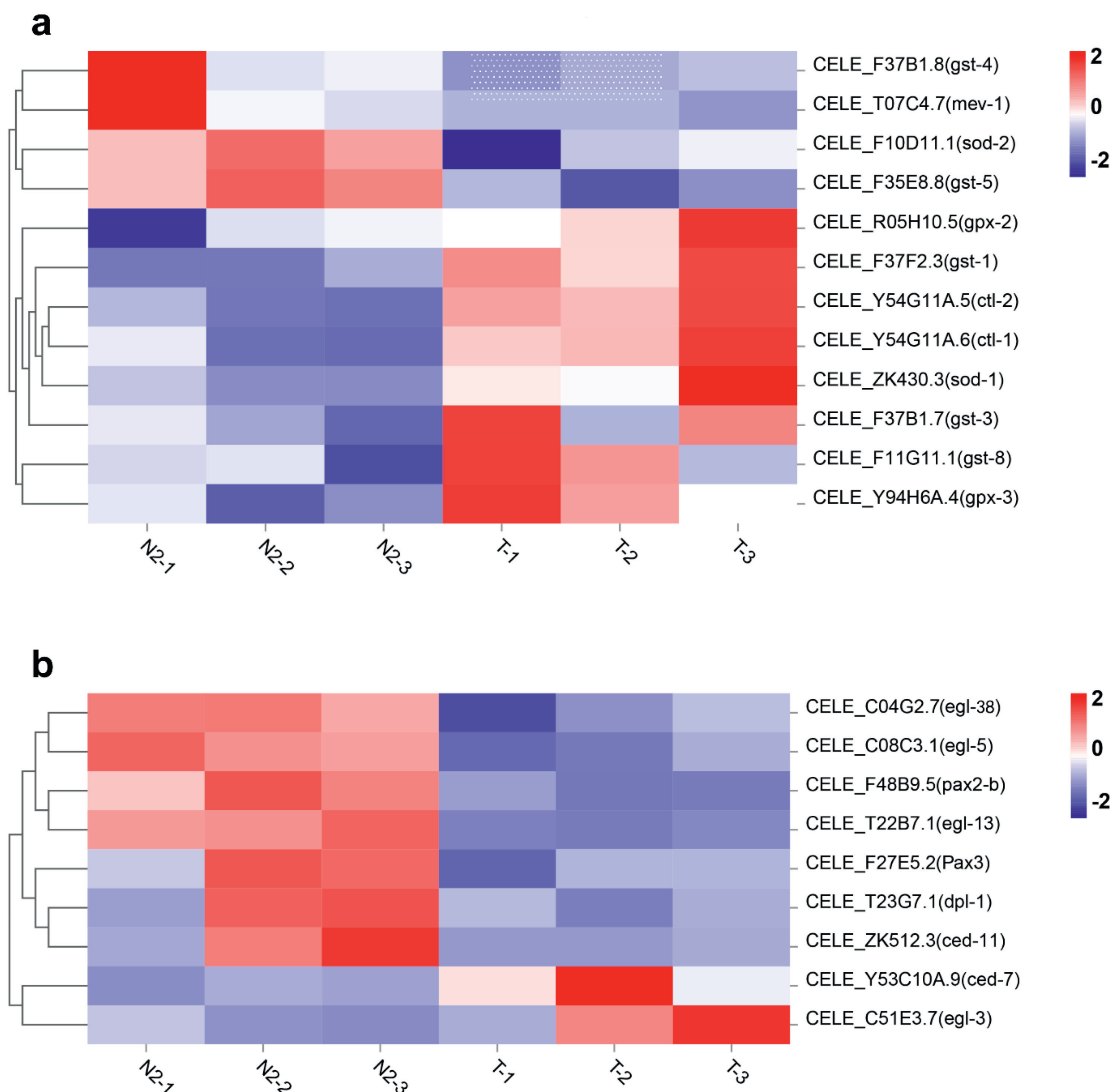

Fig. 3 – Validation of certain genes of significantly differential expression in nematodes. (a) Expression of genes related to oxidative stress and (b) expression of genes related to cell apoptosis. N2-1, N2-2 and N2-3 stand wild type in the control group, while T-1, T-2 and T-3 stand ENR-exposed nematodes. The relative expression ratio in the treatments was normalized by *z*-score (Student's *t*-test, $p < 0.05$).

lular components such as lipids and proteins, and eventually cause multiple toxic effects and/or illnesses (Fridovich, 1999; Rosenblat et al., 2002). The impaired antioxidant defense system after exposure to ENR provided further evidence that oxidative stress has an essential role in the reproductive toxicity of ENR in *C. elegans*.

2.2.3. *Partial rescue of the effects of ENR by antioxidants*

To further confirm the reproductive toxicity of ENR related to oxidative stress, two antioxidants of NAC and β-carotene were added into the tested NGM plate to observe the rescue effects, respectively. As shown in **Appendix A Fig. S6a-f**, the brood size and egg hatchability of the nematodes that were both treated with ENR and antioxidants recovered significantly as compared with only ENR-exposed nematodes (**Appendix A Fig. S6a** and b). Besides, the content of the GSH and the activity of SOD, CAT, GSH-PX show the same results (**Appendix A Fig. S6c-f**). NAC acts antioxidant activity through the targeted replenishment of GSH to quench the ROS in deficient cells (Rushworth and Megson, 2014). β-carotene molecule contains several double bonds which are susceptible to quenching the ROS such as ·OH, O_2^{-}· and RO·, thus protecting the organism from oxidative damage (Kawata et al., 2018). Despite the differences in treatment outcomes, both NAC and β-carotene could partially rescue the effects of ENR on nematodes.

NAC and β-carotene were widely used in the rescue of diseases caused by oxidative stress (Rinnerthaler et al.,

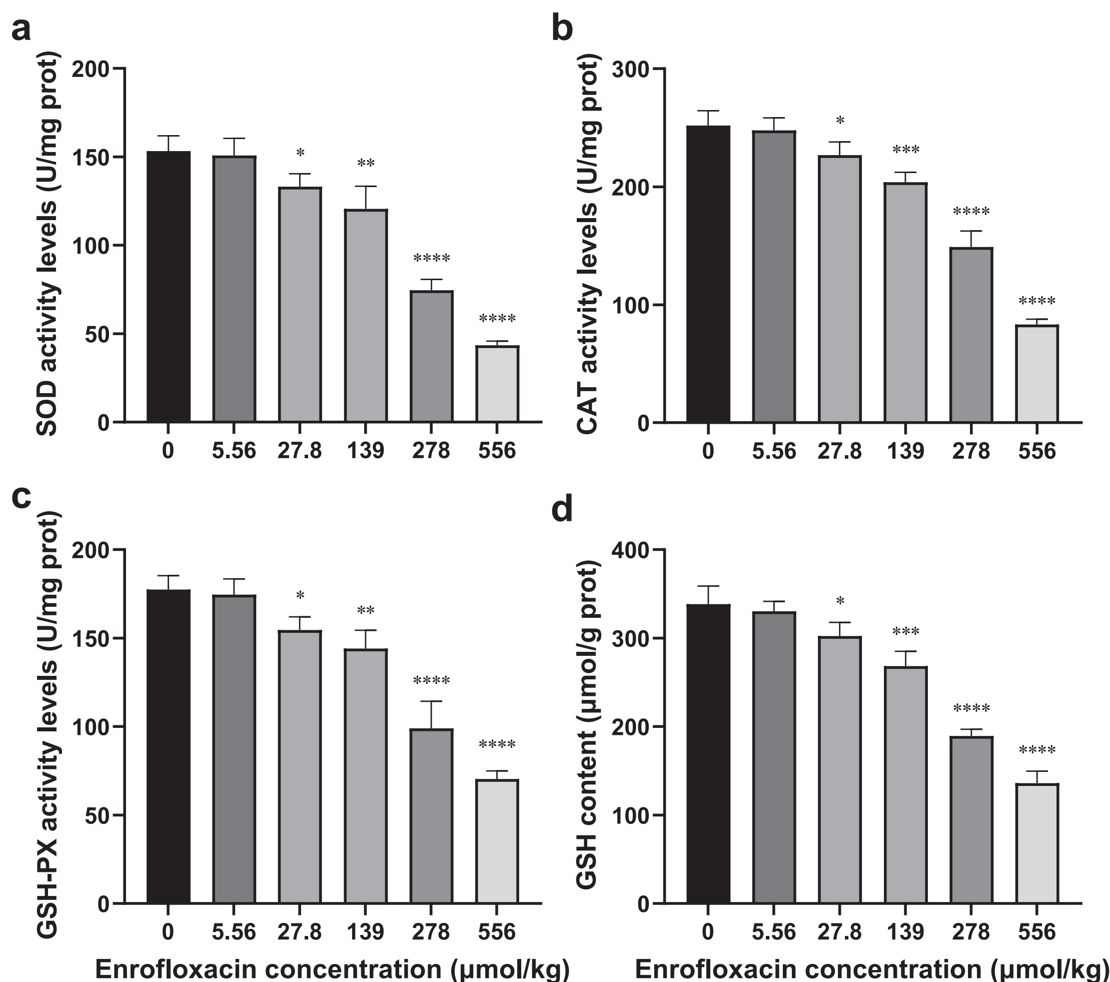

Fig. 4 – Effects of enrofloxacin (ENR) on enzymatic antioxidant systems. The activity levels of (a) SOD, (b) CAT, (c) GSH-PX and (d) GSH content were reduced in *C. elegans* after exposure to ENR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ in comparison with the value for the control (one-way ANOVA followed by Dunnett's test). Each treated concentration had four replicates.

2015; Santus et al., 2014). It has been reported that antioxidants could help to prevent oxidative stress and prolong life expectancy of *C. elegans* (Savion et al., 2018). Similarly, β-carotene could improve oocyte development and maturation under oxidative stress (Yu et al., 2019). Our results have shown the antioxidant efficacy of NAC and β-carotene in terms of reproduction and enzymatic activity in *C. elegans*, which also demonstrated that ENR-induced reproductive damage would be caused by oxidative stress.

2.2.4. *Effects of the ENR on germ cell apoptosis*

Although it was mentioned that oxidative stress caused by ENR could lead to cell apoptosis, the most straightforward and commonly used method to screen for apoptosis was to detect the cell corpses based on AO nucleic acids staining (Wang et al., 2008). As shown in Fig. 5 a, b and c, there were no or few germ cell corpses in control group and only antioxidant-exposed group, while obvious germ cell corpses were observed in ENR-exposed *C. elegans*. Exposure to 139 and 278 μmol/kg of ENR cause a significant increase in the germ cell corpses (Fig. 5 d and g). This was consistent with the above finding that the hatchability of eggs decreased with increasing ENR concentration. Interestingly, germ cell corpses were reduced or recovered partially in the nematodes that were also treated with antioxidants (Fig. 5 e, f, h, and i). Generally, the rescue effects of antioxidants further demonstrated that ENR induced nematodes germ cell apoptosis which was caused in part by oxidative stress. It has been reported that 556 μmol/kg ENR exerted cell apoptosis in grass carp for overproduction of ROS (Liu et al., 2015). Therefore, the cell apoptosis and the relevantly reproductive toxicity in *C. elegans* were associated with the oxidative stress induced by ENR.

2.3. *Environmental risk assessment*

We calculated the risk quotients (RQs) of reproductive toxicity of ENR in the soil and livestock manure samples from several countries based on ENR concentrations in the samples and the threshold value of EC_{10} in *C. elegans* for the egg hatchability (2.65 μmol/kg). The concentrations of ENR in the

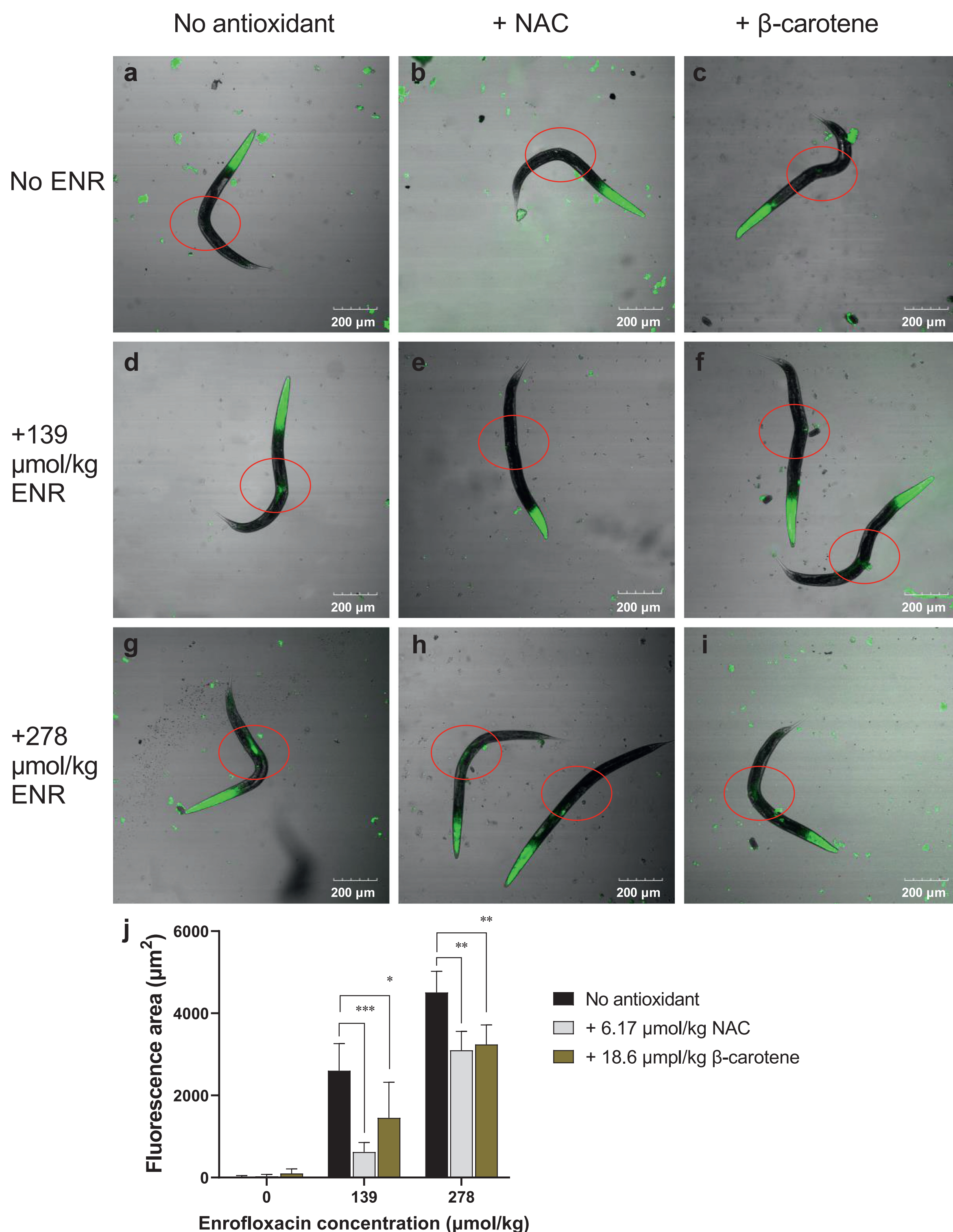

Fig. 5 – Effects of enrofloxacin (ENR) on germ cell apoptosis in nematodes. The fluorescence area in red cycle is positively correlated with the number of germ cell corpses. The fluorescence area in red cycle of the ENR-exposed groups without antioxidants was significantly larger than that of the groups with antioxidants. (a–i) Images showing the level of cell apoptosis at different exposure conditions. (j) Comparison of the fluorescence area near the vulva of nematodes. The test number of (a–j) was $n \geq 10$ worms for each treatment. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ in comparison with the value for only ENR-exposed groups (two-way ANOVA followed by Tukey's test). NAC: N-acetyl-L-cysteine.

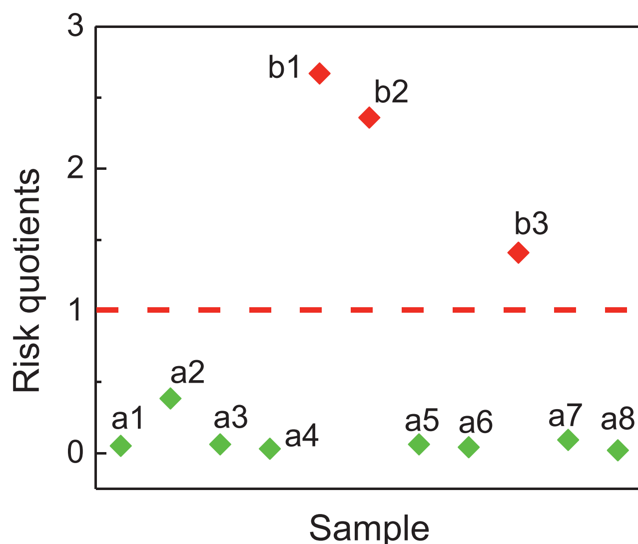

Fig. 6 – Risk quotients of reproductive toxicity in *C. elegans* exposed to enrofloxacin in the soil (a1-a8) and manure (b1-b3) samples. The red dotted line represents a risk quotient of 1, and a value greater than 1 indicates that enrofloxacin in the sample may cause reproductive toxicity.

samples referred to the references (Karcı and Balcıoğlu, 2009; Martínez-Carballo et al., 2007; Sturini et al., 2012; Leal et al., 2012; Zhou et al., 2020; Li et al., 2011). Results showed that RQs were ranged from 0.021–0.39 in soil from North part of Marmara Region, Turkey (a1), Austria (a2), South Lombardy, Italy (a3) and São Paulo State, Brazil (a4), in some pig manure and pig manure-based fertilizer soils from Jiangsu, China (a5 and a6), and in traditional/green food farmland soils form the Pearl River Delta area, China (a7 and a8) (Fig. 6). Although the RQs in those samples were below 1, it should be noted that ENR was the only antibiotic used for evaluation in this manuscript. In fact, the soil contains a variety of FQ antibiotics and other antibiotics, such as β-lactams, macrolides, sulfonamides and tetracyclines. The total concentration of various FQs in soil might be high (Li et al., 2021). Moreover, the RQs in some chicken and duck manures from Jiangsu, China (b1 and b2) and in farmland soils affiliated with livestock from the Pearl River Delta area, China (b3) were ranged from 1.41–2.67 ($>$ 1) (Fig. 6), indicating a high reproductive risk for ENR in these samples.

As well, the management of antibiotics-containing domestic animal wastes faces various problems due to the limited infrastructure. There are so far no specific treatment requirements for livestock wastes before discharge in China, especially in majority of the rural areas (Zhang et al., 2015). The results from the soils and the livestock manures, especially the farmland soils affiliated with livestock (Fig. 6, b3, RQ = 1.41) suggested that there were potential risks in livestock manure-fertilized agricultural soils for the presence of FQs. The reproductive toxicity caused by FQs could reduce the number of nematodes, decrease the nutrient cycling and plant growth in the agricultural soils, and maybe further reduce the soil sustainability.

3. Conclusion

In the present study, the reproductive toxicity of FQs on the nematode was investigated by the representative of ENR. The concentration-effect curves showed that the EC_{50} of ENR on the reproductive toxicity outcomes of brood size and hatchability of egg were (494.3 ± 272.9) µmol/kg and (107.4 ± 30.9) µmol/kg, respectively. The changes of the antioxidant enzyme activity, the differential expressions of the genes associated with oxidative stress and cell apoptosis, and the increases of germ cell apoptosis with the exposure concentration of ENR demonstrated that germ cell apoptosis under oxidative stress has a key role in ENR-induced reproductive toxicity in nematodes. RQs of soil and livestock samples from several countries have been calculated based on the threshold value of EC_{10} for the outcomes of egg hatchability in *C. elegans* (2.65 µmol/kg). The results indicated possible reproductive toxicity on the nematodes in certain agricultural soils for the presence of FQs. This is the first study suggested that chronic exposure to FQs at certain levels in environment would induce reproductive toxicity to the nematodes and might reduce the soil sustainability, alarming the environment risks of antibiotics abuse.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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Appendix A Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jes.2022.07.002.

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