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Effect of enrofloxacin on the proteome of earthworms

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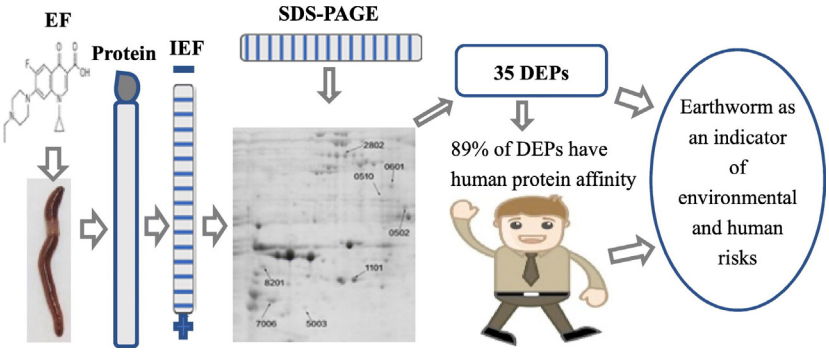
HIGHLIGHTS

- Earthworms were exposed to enrofloxacin, their proteomic response was studied.
- 35 differentially expressed proteins (DEPs) from four functional groups were found.
- 89% of *E. fetida* DEPs have sequence homologies with corresponding human proteins.
- Earthworm proteomic data may be predictive of environmental and human health risks.

GRAPHICAL ABSTRACT



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ABSTRACT

The environmental and human health risks of veterinary drugs are becoming public health issues. Enrofloxacin (EF) is an extensively used animal-specific antibacterial agent that leaves drug residues in the environment. This study investigated the proteomic response of the earthworm *Eisenia fetida* to EF exposure. Earthworms were exposed to EF in soil at 1–500 $mg \cdot kg^{-1}$, and samples were collected at intervals during a 28 day period. The extracted proteins were separated by two dimensional electrophoresis to detect differentially expressed proteins (DEPs) in EF-exposed earthworms. In total, 35 unique DEPs were found. These proteins were subjected to MALDI-TOF/TOF-MS analysis and identified through comparison of their mass spectra with those in protein databases. The DEPs were grouped on the basis of their function, into metabolism, stress-related, transport, transcription, and predicted/hypothetical protein categories. Knowledge of proteins that are induced or repressed by EF in earthworms could provide insight into mechanisms of sub-clinical physiological effects of xenobiotic residues in the environment, and may also help understand synergy between pollutants. As several DEPs in *E. fetida* showed similarity to human protein sequences, *E. fetida* has potential as an indicator species to assess the environmental and biological risks of drug residues.

1. Introduction

Enrofloxacin (EF) is one of the most extensively used animal-specific antibiotics because of its broad spectrum of activity (Brown, 1996). Therefore, large amounts of EF and its metabolites have been discharged into the environment (Huang et al., 2000; Li et al., 2016; Wei and Li,

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2007). Although EF is an animal-specific drug, it has been detected in the body fluids of Chinese children (detection rate of 4.2%, average concentration 2.1 $mg \cdot l^{-1}$) (Wang et al., 2015). This has raised concerns about the potential impact of these residues on human health, particularly on growth and development during childhood, and has emphasized the importance of assessing the human health risks of EF in the environment.

As a key representative of soil fauna, earthworms are essential for maintaining soil fertility through their burrowing, ingestion, and excretion (Pérès et al., 2011). Earthworms are also increasingly recognized as indicators of agroecosystem health and as an ecotoxicological sentinel species because they are constantly exposed to soil contaminants and are sensitive to environmental changes (Gong and Perkins, 2016; Tang et al., 2013; Zhang et al., 2013). Pharmaceutical residues in soils can be taken up and accumulated by earthworms (Carter et al., 2016; Kinney et al., 2008). Several studies have described the responses of earthworms to antibiotics, including EF. The growth rate of the earthworm *Eisenia fetida* exposed to EF at 5000 $mg \cdot kg^{-1}$ decreased by approximately 64%, and catalase activity varied depending on the EF concentration, the exposure time, and the tissue tested (Gao et al., 2008). A high EF concentration (>1 $mg \cdot kg^{-1}$) in soils was shown to inhibit the growth and reproduction of *E. fetida* (Li et al., 2015). Even low concentrations of EF (0.01 $mg \cdot kg^{-1}$) decreased the abundance of soil microbes (Ma et al., 2007). Interestingly, environmentally relevant concentrations of EF were found to enhance both the uptake and the toxicity of cadmium in earthworms (Li et al., 2016). Dong and coauthors observed that tetracycline and chlortetracycline induced significant DNA damage and biochemical toxicity in *E. fetida* (Dong et al., 2012).

Several studies have focused on the effects of toxins on the proteome of various invertebrate models (Dunbar et al., 1990; Gutierrez-Carbonell et al., 2013; Shevchenko et al., 1996) and ecotoxicoproteomic analyses have been conducted for earthworms (Wang et al., 2010b). However, the effects of veterinary drugs on the earthworm proteome are unknown. Proteomic analyses are useful to unravel the effects of stresses on metabolic processes (Jorrín-Novo et al., 2009), and provide a powerful new method to assess toxicology (Snell et al., 2003). This approach has been used to study the effects of cadmium exposure on *E. fetida* (Wang et al., 2010b), but the effects of EF on the proteome of *E. fetida* are unknown. Qi and coauthors reported that differentially expressed proteins (DEPs) in *Escherichia coli* exposed to EF were principally involved in antibiotic resistance and were related to the oxidative stress response (Qi et al., 2013). Since EF is an inhibitor of DNA gyrase, the overexpression of a DNA starvation/stationary phase protection protein could be important to counteract the effects of EF (Piras et al., 2015). The authors are not aware of any previous studies of the effects of EF on the proteome of other organisms.

We conducted a proteomic analysis to identify DEPs in the earthworm *E. fetida* on exposure to EF. The objectives were as follows: 1) to identify the proteins involved in the earthworm response to EF in order to explore the mechanism of EF toxicity, and 2) to explore the potential to use *E. fetida* as an indicator species for detection of veterinary drug residues in the environment.

2. Materials and methods

2.1. Chemicals and reagents

Enrofloxacin (99%), trichloroacetic acid (TCA), lauryl sodium sulfate (SDS), dithiothreitol (DTT), urea, and iodoacetamide were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetone, absolute ethyl alcohol, ammonium persulfate (AP) methanol, glacial acetic acid, and glycerol were obtained from Sangon Biotech (Shanghai, China). Trizol RNA Extraction Reagent was purchased from TransGen Biotech (Beijing, China). Immobilized pH gradient (IPG) strips (17 cm, linear pH 4–7), 2-dimensional electrophoresis buffer, the iScriptTM cDNA Synthesis Kit, and iQTM SYBR Green Supermix were purchased from Bio-Rad (Hercules, CA, USA).

The protein extraction buffer consisted of 1 g TCA and 100 mg DTT dissolved in 10 ml acetone. The balance stock solution was prepared by adding 36 g urea, 2 g SDS, 25 ml 1.5 M pH 8.8 Tris-HCl, and 30 ml glycerol to distilled water and making up the volume to 100 ml. Aliquots of this stock solution (10 ml) were stored at −20 °C until use. Balance buffer A was prepared by dissolving 100 mg DTT in 10 ml balance stock solution. Balance buffer B was prepared by dissolving 250 mg iodoacetamide in 10 ml balance stock solution. The SDS electrophoresis buffer consisted of 250 mM Tris (pH 8.3), 1.92 M glycine, and 1% (*w*/*v*) SDS. To prepare the Coomassie brilliant blue staining solution, 1.25 g R-250 was dissolved in a mixture of 400 ml methanol, 100 ml glacial acetic acid, 500 ml ultrapure water, and the mixture was filtered to remove particulates. The decoloring solution consisted of 1:1:8 methanol:glacial acetic acid:ultrapure water.

2.2. Earthworms, soil, and EF exposure

We purchased *E. fetida* from a commercial earthworm farm in Shanghai and kept the animals in test substrates for 1 week before the experiment. Soil was collected from the campus of Shanghai Jiao Tong University, from the top 20-cm layer after removing vegetation. The soil used for the experiment, and the earthworms had had no previous exposure to pharmaceutical residues. As a precaution, we also measured the EF content of both test soil and earthworms, and no EF was detected. The soil was homogenized, air-dried, and sieved before use. The soil physicochemical properties were as follows: pH, 7.5; EC, 129.5 $\mu S \cdot cm^{-1}$; organic matter, 32.1 $g \cdot kg^{-1}$; total P, 0.958 $g \cdot kg^{-1}$; total N, 1.85 $g \cdot kg^{-1}$; total K, 2.53 $g \cdot kg^{-1}$. The EF was dissolved in methanol and spiked into the soils to final concentrations of 0, 1, 10, 100, 500 $mg \cdot kg^{-1}$ dry soil, with five replicates per treatment. The concentrations of EF typically reach 0.1 $mg \cdot kg^{-1}$ background in the environment, 1 mg kg^{-1} in grazed areas, and sometimes as high as 10 $mg \cdot kg^{-1}$ soon after the application of animal manure (Tai et al., 2012; Zhao et al., 2010). The 100 and 500 $mg \cdot kg^{-1}$ concentration treatments were included in order to observe the toxicological response at a high dose exposure. Such concentrations might occur following accidental spillage or deliberate illegal discharge of farm or pharmaceutical factory wastewater, or farm wastes such as poultry litter with high EF concentration. The soils were placed in a fume hood for three days and occasionally stirred, to allow methanol to entirely evaporate. Pure water was added to the soils to keep the soil humidity at 70% of maximum water holding capacity. Adult earthworms with a clitellum (each worm weighing 0.49–0.54 g) were added to soils, and then the soils were placed in a dark room at 20 °C. The earthworms were sampled on days 1, 3, 7, 14, 21, and 28 in each treatment. The earthworms were kept on filter papers soaked with isotonic balanced salt solution at 22 ± 1 °C for 2 days to void the gut load. Proteins were extracted from the freshly depurated earthworms. The earthworms used for mRNA extraction were frozen in liquid nitrogen and stored at −80 °C until used. Parts of depurated earthworms were used to analyze the EF content.

Proteins were extracted using the trichloroacetic acid-A (TCA-A) procedure (Damerval et al., 1986; Wang et al., 2010c; Wu et al., 2013) with slight modifications. Briefly, two depurated fresh earthworms were weighed, and then ground in a pre-chilled mortar with nine volumes of protein extraction buffer (*w*/*v*). The mixture was stored at −20 °C overnight, and then centrifuged at 15000*g*, 4 °C, for 30 min. The pellet was suspended in pre-chilled acetone containing 1% DTT, kept at −20 °C for 1 h, and then centrifuged at 15000*g*, 4 °C, for 30 min. The supernatant was removed and the tube was placed in a fume hood to allow the acetone to evaporate. The dried pellet was re-dissolved in 2-D buffer (Bio-Rad), and then centrifuged at 15000*g*, 4 °C, for 1 h. The protein concentration in the supernatant was determined using a Bradford Protein Assay Kit (Sangon Biotech, Shanghai,