The Toxicity Assessment of ethyl \(p\)-hydroxybenzoate in Nematode \(C.\) \textit{elegans}

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Abstract

Ethyl paraben (EP) is an antiseptic commonly used in food, medicine, cosmetics, and children's products. Parabens are popular due to their broad antibacterial spectrum, low cost, and stable pH characteristics, and though once considered safe as well as effective, recent studies have shown that EP has considerable oestrogenic activity which has adverse effect on the human reproduction system. In our study, we used a \textit{Caenorhabditis elegans} assay system to investigate EP toxicity and its underlying mechanism. We found that prolonged exposure to EP decreased body length and locomotion behavior in the nematodes. Furthermore, most of the toxicities were transferable. Locomotion behavior defects were only partially recovered in progeny, and higher concentrations caused more significant defects; locomotion behavior improved after subsequent exposure to mutants PMK-1 and PMK-3. Taken together, the results showed that EP exerts adverse effect on \textit{C. elegans} and may induce toxicity through various underlying mechanisms.

Keywords: Ethyl \(p\)-hydroxybenzoate, \textit{Caenorhabditis elegans}, toxicity

1. Introduction

Parabens are a group of phenol preservatives (Arif & Handan, 2013) that are commonly used in food (Soni et al., 2001; 2002), cosmetics, and drug industries, as well as in various children's products (Elder, 1984). Parabens mainly include methyl \(p\)-hydroxybenzoate (MP), ethyl \(p\)-hydroxybenzoate (EP), propyl \(p\)-hydroxybenzoate (PP), and butyl \(p\)-hydroxybenzoate (BP). EP is the most commonly used among them. Given their wide range of microbial and antifungal activity, their safety (e.g., low acute toxicity, low irritating or sensitising potential,) and their stability over a large pH range, parabens have replaced the majority of sodium benzoate food preservatives in China.

Recent studies have shown that parabens have estrogenic effects, and that the damage to the reproductive system is dose-dependent. After long-term exposure to parabens, rats showed decreased hormone levels, endocrine disorders, abnormal reproductive organ development; paraben exposure dramatically reduced the male rat sperm count and sperm vitality and the weight of postnatal rats (Oishi, 2002). Recent studies have also shown that the metabolic pathways of these substances are mainly blood and urine with relatively little accumulation. Other studies have identified parabens in breast cancer samples, shown that they can induce breast cancer cells, and identified cumulative effect, altogether suggesting that parabens pose a threat to human health. There has been a general lack of toxicological research on parabens in vivo, however.

The nematode \textit{Caenorhabditis elegans} (\textit{C. elegans}) is a classical model animal widely used in biomedical and toxicological research (Leung et al., 2008; Zhao, Wu, Li, & Wang, 2013; Zhao & Wang, 2012). Due to it has invariant and fully described developmental program, well-characterized genome, short and prolific life cycle, and small body size (Leung et al., 2008; Zhao, Wu, Li, & Wang, 2013), \textit{C. elegans} is commonly utilized in toxicity research and in developing new drugs (Wu, He, Liu, Li, & Wang, 2011; Jiang et al., 2011; Wu, Qu, Li, & Wang, 2012; Li, Wang, Wu, Li, & Tang, 2012; Yu, Chen, Zhang et al., 2013; Leung et al., 2008). Body length and locomotion behavior are the indices typically used to measure toxic effects on \textit{C. elegans}: Body length, which reflects the overall health level of \textit{C. elegans}, is often used to characterize the toxicity of heavy metals (Yu et al., 2013; Boyd et al., 2003), and locomotion behavior, which reflects the overall effect of \textit{C. elegans} nerves and muscles, is often used to measure toxic effects in heavy metals, drugs, and other substances (Yu et al., 2013; Leung et al., 2008; Gray, Hill, & Bargmann, 2005). To date, \textit{C. elegans} has been successfully applied to a wide variety of toxicological research projects (Traunspurger et al., 1997).
In the present study, our primary objective was to determine the feasibility of using *C. elegans* for evaluating EP toxicity. We hope that the results presented below will be helpful for future toxicological research and provide a better understanding of the safety of EP or even antiseptic phenols.

2. Materials and Methods

2.1 Reagents and *C. elegans* Strain Preparation

EP is obtained from Sigma-Aldrich (St. Louis, MO, USA), and its purity is 99.9%, it was dissolved in Dimethyl sulfoxide solution and then diluted by ddH2O. Control was also with the same concentration of DMSO. Nematodes used were wild-type *C. elegans* N2, mutants of pmk-1, pmk-3. originally obtained from Caenorhabditis Genetics Center, was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described.

Gravid nematodes were washed off the plates into centrifuge tubes, and were lysed with a bleaching mixture (0.45 M NaOH, 2% HOCl). Age synchronous populations of L1-larvae nematodes were obtained by the collection (Donkin & Dusenbery, 1993) Nematodes were washed with a modified K medium (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5). Exposures were performed from L1-larvae to adult (prolonged exposure) in K medium of 12-well sterile tissue culture plates at 20°C incubator in the presence of food.

2.2 Lethality and Growth

Lethality was evaluated by the percentage of survival animals. Following exposure, inactive ones were scored under a dissecting microscopy and nematodes were judged to be dead if they did not respond to stimulus using a small, metal wire. One hundred nematodes were examined per treatment. Growth was assessed by the body length, which was determined by measuring the flat surface area of nematodes using the PS. Ten nematodes were examined per treatment. Three replicates were performed.

2.3 Locomotion Behavior

Locomotion behaviors of nematodes were evaluated by head thrash and body bend. To assay head thrash, every examined nematode was transferred into a micro titer well containing 60 μl of modified K medium on the top of agar, and head thrashes were counted for 1-min after a 1-min recovery period. A thrash was defined as a change in the direction of bending at the mid body. To assay body bend, nematodes were picked onto a second plate and scored for the number of body bends in an interval of 20 sec. A body bend was counted as a change in the direction of the part of the nematodes corresponding to the posterior bulb of the pharynx along the y axis, assuming that the nematode was traveling along the x axis. Twenty nematodes were examined per treatment. Three replicates were performed.

2.4 Statistical Analysis

All data in this article were expressed as means ± standard error of the mean (S.E.M.). Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance (ANOVA). Probability levels of 0.05 and 0.01 were considered statistically significant.

3. Results

3.1 Comparison of Lethality in EP Exposed Nematodes

![Figure 1](https://www.ccsenet.org/ijb/International-Journal-of-Biology-Vol-8-No-3-2016/39.png)

Figure 1. Comparison of lethality in nematodes exposed to different concentrations of EP

Exposures were performed from L1-larvae to adult (prolonged exposure). Thirty nematodes were examined per treatment for lethality assay. Bars represent mean ± S.E.M. **P<0.01.**
Considering the fact that many toxicants at the low concentrations may have the adverse effects on nematodes after prolonged exposure (Zhao, Wu, Li & Wang, 2013) (Xing & Wang, 2009) (Li et al., 2012) (Li et al., 2012) (Wu, Li, Tang, & Wang, 2012) (Wu et al., 2013) (Wu et al., 2013) we performed the prolonged exposure for EP. Lethality is the important indexes for evaluation of toxicity, it will be effect after exposed all kinds of toxicants. Concentrations of 100–300 μg/ml were used for prolonged exposure to EP. After prolonged exposure from L1-larvae to the adult stage, EP did not induce lethality of nematodes (Figure 1).

3.2 Comparison of Growth in EP Exposed Nematodes

Body length as evaluation index for development of nematodes, which reflects the change of the C. elegans ontogenesis rate and physiological state. It provide premise for using the C. elegans developmental toxicology studies.

We performed the prolonged exposure for EP. Concentrations of 100–300 μg/ml were used for prolonged exposure to EP. After prolonged exposure from L1-larvae to the adult stage, EP at concentrations more than 200 μg/ml significantly reduced body length of nematodes (Figure 2).

![Figure 2. Comparison of growth in nematodes exposed to different concentrations of EP](image)

Exposures were performed from L1-larvae to adult (prolonged exposure). Ten nematodes were examined per treatment for growth assay. Bars represent mean ± S.E.M. *P<0.05, **P<0.01.

3.3 Comparison of Locomotion Behavior in EP Exposed Nematodes

Neuron may be important secondary targeted organs for toxicants in nematodes (Zhao, Wu, Li, & Wang, 2013) (Wu et al., 2013) (Li et al., 2012) (Wu, Yin, Li, Tang & Wang, 2013), we evaluated the possibly toxic effects of EP exposure on the locomotion behavior of nematodes. Head thrashes and body bends were used to assay the locomotion behavior of C elegans (Roh & Choi, 2008).

Prolonged exposure to 100 μg/ml of EP did not significantly influence locomotion behavior (head thrashes and body bends) of nematodes. However, prolonged exposure to EP at concentrations more than 200 μg/ml significantly reduced locomotion behavior (head thrashes and body bends) of nematodes (Figure 3).

![Figure 3. Comparison of locomotion behavior in nematodes exposed to different concentrations of EP.](image)

Exposures were performed from L1-larvae to adult (prolonged exposure). Ten nematodes were examined per treatment for locomotion behavior assay (head thrashes and body bends). Bars represent mean ± S.E.M. *P<0.05, **P<0.01.
3.4 Toxic Effects of EP on the Locomotion Behaviors of their Progeny

According to current work, EP exposure resulted in a remarkable toxicity on locomotion behavior. We further analyzed the toxic effects of EP on locomotion behaviors in progeny. Investigation on their progeny further indicates that both the body bends defects and the head thrashes defects could be rescued only to very limited degrees in nematodes. Both the body bends and the head thrashes in progeny from animals exposed to 300μg/ml EP still showed significant defects compared to control (Figure 4).

![Figure 4](https://example.com/figure4.png)

Figure 4. Toxic effects of EP on the locomotion behaviors of their progeny. (A) Progeny of animals exposed to EP on locomotion behavior as indicated by head thrashes. (B) Progeny of animals exposed to EP on locomotion behavior as indicated by body bends.

To evaluate the EP toxicity in progeny, eggs of animals exposed to EP were transferred to a normal NGM plate without the addition of EP solution. Ten nematodes were examined per treatment for locomotion behavior assay (body bends and the head thrashes). Bars represent mean ±S.E.M. *P<0.05, **P<0.01.

3.5 Molecular Mechanism for EP Toxicity

![Figure 5](https://example.com/figure5.png)

Figure 5. Locomotion behaviors in wild-type and mutants exposed to EP. (A) Effects of EP on locomotion behavior in pmk-1 and N2 as indicated by head thrashes and body bends. (B) Effects of EP on locomotion behavior in pmk-3 and N2 as indicated by head thrashes and body bends

Exposures were performed from L1-larvae to adult (prolonged exposure) at the concentration of 300 μg/ml. Ten nematodes were examined per treatment. Bars represent mean ±S.E.M. *P<0.05, **P<0.01.

To further confirm the functions of the dysregulated genes in regulating the toxicity formation from EP. We used the corresponding mutants to investigate the locomotion behavior of these mutants exposed to EP. It is known that mitogenactivated protein kinases (MAPKs) serve as transducers of extracellular stimuli, which play key
roles in diverse physiological processes, including stress response (Kyriakis & Avruch, 2001). A C. elegans ortholog of the p38 MAPK, pmk-1 is known to play a critical role in the response against oxidative stress and innate immunity in C. elegans (Troemel et al., 2006) (An et al., 2005). Although the roles of the pmk-1 p38 MAPK pathways in various stress responses of C. elegans have already been reported (Wang S, Wu L, Wang Y, Luo X & Lu Y. 2009) (Shivers et al., 2010), they have rarely been approached in an ecotoxicological context.

Interestingly, we found that compared with wild type the pmk-1 and pmk-3 mutants are more resistant to the toxic effect of EP from locomotion behavior (body bends and head thrashes) (Figure 5). These data further confirm the involvement of the P38 signaling pathways in regulating the toxicity formation from EP.

4. Discussion

In this study, we established a basis for toxicological research on EP in vivo. We measured the body length of C. elegans to detect the toxicity from prolonged exposure to EP.

We did not observe the induction of lethality in nematodes after EP exposure at the concentrations we examined (Fig 1). Although EP exposure did not affect survival rate, it was likely to affect other indicators.

After the detection of parabens in human breast cancer tissue, the relationship between cancer and parabens has been the subject of intensive research. Recent studies have shown that long-term paraben exposure increases in the incidence of breast cancer, impacts human reproductive functions, and creates estrogenic stimulus in malignant melanoma (Darbre & Harvey, 2008; Martin et al., 2010). These results have, of course, created a great deal of anxiety regarding the safety of parabens as antimicrobial preservatives.

EP had a significant impact on the locomotion and body length of wild-type C. elegans, suggesting that EP has the potential for neural toxicity and that exposure affected the nematodes’ development (Figs. 2-3). Whether the toxicity caused by EP was transferable to progeny was not clear, so it was necessary to systematically analyze the toxicity data to confirm possible transfer in a specific model organism. As shown in Figure 4, the locomotion behavior defects caused by EP exposure was largely transferrable to progeny nematodes.

In C. elegans, several signaling cascades are involved in the response to abiotic stressors. The most important are mitogen-activated protein kinases (MAPKs) such as the p38, JNK, or ERK MAPK, which are conserved signaling proteins that fulfill various functions (Sakaguchi, Matsumoto & Hisamoto, 2004). MAPK pathways are composed of MAPK kinase kinases (MAP3Ks), MAPK kinases (MAP2Ks), and MAPKs. PMK-1 and PMK-3 are C. elegans p38 MAPK homologues (Berman et al., 2001).

PMK-1 is part of an operon comprising three homologues of the mammalian p38 MAPK. Berman et al. (2001) suggested that PMK-1 expression is transcriptionally regulated by an operon promoter upstream of PMK-3. PMK-1 also participates in stress responses to biotic stressors (Kim et al., 2002; Shivers et al., 2010), which is why the gene for the most functional protein lies downstream of two less important p38 MAPK homologues. We found that compared with the wild type, the locomotion behavior of mutant PMK-1 and PMK-3 animals improved. In effect, the P38 signaling pathway was involved in the toxicity.

EP toxicity and food safety is a crucial research topic in regards to the potential threats to human health due to paraben exposure. As discussed above, EP has shown estrogenic activity, which makes its toxicity and related mechanism very important. In this study, we demonstrated that the C. elegans assay system can be utilized to successfully measure the in vivo toxicity from food additives such as EP. We hope that our findings regarding the multiple toxicities of EP in nematodes prove helpful for further understanding of the mechanism of EP toxicity; the potentially transferable properties of the multiple defects caused by EP exposure are especially interesting, and merit further research.

Author Contributions

Conceived and designed the experiments: TX LY. Performed the experiments: TX. Analyzed the data: TX. Contributed reagents/materials/analysis tools: LY. Wrote the paper: TX LY.

References


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