The chronic toxicity of bisphenol A to Caenorhabditis elegans after long-term exposure at environmentally relevant concentrations

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HIGHLIGHTS

• Long-term exposure was conducted to evaluate chronic toxicity of BPA on Caenorhabditis elegans.
• Long-term exposure significantly decreased the head thrash frequency at 0.001 μM.
• Chronic exposure induced a stronger stress response than prolonged exposure.
• The gene cep-1 might act as an important role in BPA-induced chronic toxicity.

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Abstract

To investigate biological effects of bisphenol A (BPA) over the long term, the model animal Caenorhabditis elegans was used to conduct the chronic exposure. C. elegans were exposed to BPA (0.0001–10 μM) from L4 larvae to day-10 adult in the present chronic toxicity assay system. Multiple endpoints at the physiological (growth, locomotion behaviors and lifespan), biochemical (lipofuscin accumulation), molecular (stress-related genes expressions), and population (population size) levels were examined. At the physiological level, BPA exposure induced significant negative effects on the indicators. Among the endpoints, head thrash was most sensitive and the detection limit was 0.001 μM. At the biochemical level, BPA exposure induced no significant effects on lipofuscin accumulation. At the molecular level, BPA induced strong stress responses in vivo. At the population level, the population size was significantly decreased in the treatment groups from 0.1 to 10 μM. Compared to the previous short-term toxicity evaluation, long-term exposure to BPA induced a more obvious response at the same concentration, and the phenomenon might be due to cumulative toxic effects. By the Pearson correlation analyses, cep-1 was speculated to act as an important role in BPA-induced chronic toxicity on C. elegans.

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1. Introduction

Bisphenol A (BPA) is a high-volume chemical that is extensively used to make polycarbonate plastics, epoxy resins, and plastic consumer products (Hoekstra and Simoneau, 2013). As a known
endocrine disruptor, BPA could cause various effects including developmental toxicity (Lombó et al., 2015), reproductive toxicity (Sieratowicz et al., 2011), neurotoxicity (Wang et al., 2015), mutagenicity (Lee et al., 2013) and immunotoxicity (Lee and Lim, 2010).

In addition, the literature to date provides growing support that environmental BPA exposure could be harmful to humans, especially in regards to negative effects on children (Rochester, 2013). Because of its high production of exceeding 3.8 million tons annually and wide use in consumer products (Michałowicz, 2014), the environmental safety of BPA has been gaining more and more attentions.

More than 1 million pounds of BPA are estimated to be released into the environment annually through air, sludge, industrial wastewater, and leachate from landfills (Erler and Novak, 2010; Fu and Kawamura, 2010). Although BPA is easily degradable, it is ubiquitous in the environment owing to its continuous input (Flint et al., 2012). Therefore, biotas in ecosystems are exposed to BPA for a long time, even a whole life. Future toxicity evaluation of toxicants employing invertebrates by long-term and low-concentration exposures has already been recommended (Baun et al., 2008). To address the problems, the model animal *Caenorhabditis elegans*, a representative species of nematodes, was chosen as an excellent candidate for ecotoxicological evaluations. Due to its advantages of short life cycle, low cost, convenient handling, and high sensitivity, *C. elegans* has been successfully used to evaluate the chronic toxicity of toxicants at environmentally relevant concentrations (Li et al., 2012; Wu et al., 2012b, 2012c).

An earlier study evaluated the toxic effects of Fe2O3-NPs on *C. elegans* using 3 different assay systems: acute exposure (24-h exposure), prolonged exposure (72-h exposure), and chronic exposure (10-d exposure) (Wu et al., 2012a). The result showed that the long-term exposure could induce adverse effects on the exposed *C. elegans* at much lower concentrations compared to the short-term exposure. In our previous studies, we have evaluated the ecotoxicity of BPA to *C. elegans* by acute exposure (24-h exposure from L4 larvae), prolonged exposure (72-h exposure from L1 larvae) (Zhou et al., 2016b), and multigenerational exposure (across 4 generations) (Zhou et al., 2016a). The evaluation indicated that BPA exposure could have toxic effects on physiological traits at concentrations above 0.01 μM, and imposed developmental, reproductive and neurobehavioral toxicities on *C. elegans*. Considering the fact that the non-effect doses of BPA in the short-term toxicity tests could still be toxic to nematodes, long-term exposure of *C. elegans* to low-concentration BPA (0.0001–10 μM) was conducted in the present study, to investigate the chronic toxicity and environmental effects of BPA.

The specific development stage from L4 larvae to day-10 adult (approximately 10 days) was selected in the present assay system, according to the chronic toxicity testing method established by Shen et al. (2009). Ecotoxicological evaluations were conducted using *C. elegans* upon the physiological (growth, locomotion behaviors and lifespan), biochemical (lipofuscin accumulation), and molecular (stress-related genes expressions) responses. The specific stress-related genes included heat shock proteins (hsp-16.1, hsp-16.2, and hsp-16.48), vitellogenin (vit-2 and vit-6), xenobiotic metabolism enzyme (cyp35a2), superoxide dismutases (sod-1 and sod-2), catalase 2 (ctl-2), abnormal dauer formation proteins (daf-12 and daf-21), aging alteration protein (age-1), tumor suppressor and apoptosis proteins (cep-1 and ape-1), as well as metallothioneins (mtl-1 and mtl-2). In addition, the population-level effects of BPA exposure were also evaluated in the present study. At last, the transgenic nematode TJ375 with the GFP based reporter hsp-16.2 was attempted to be used as a biosensor to monitor the stress response.

## 2. Materials and methods

### 2.1. *C. elegans* strains preparation and BPA exposure protocols

Populations of *C. elegans* were grown on nematode growth medium (NGM) plates which were seeded with *Escherichia coli* strain OP50 as previously described (Brenner, 1974). Unless otherwise stated, the wild-type N2 Bristol strain was used in all the experiments. Transgenic line used in this study was the strain TJ375, expressing green fluorescent protein (GFP) reporter driven by the heat shock promoter hsp-16.2. Both strains were obtained from the *Caenorhabditis Genetics Center* (CGC). The age-synchronized populations of L4 stage were obtained by an alka-line bleach solution (Stiernagle, 2006), and the synchronized populations were washed with K medium (32 mM KCl, 51 mM NaCl) (Williams and Dusenberg, 1990) before treatment.

* C. elegans mainly lived in the liquid phase of soils, so liquid exposure was employed in this study. BPA (purity > 99%), from Aladdin Industrial Corporation (Shanghai, China), was dissolved in 100% ethanol to prepare desired stock solutions of BPA, and then stock solutions were diluted with K medium to get the treatment solutions with a final ethanol concentration of 0.1%. Exposures were performed in 6-cm petri dishes with 10 mL of treatment solutions. Each petri dish contained approximately 300 nematodes. For the purpose of this study, seven nominal BPA concentrations were used: 0 (control), 0.0001, 0.001, 0.01, 0.1, 1, and 10 μM. The entire assay was independently repeated four times. The controls presented in this paper were solvent controls. *C. elegans* were exposed to BPA in K medium from L4 larvae to day-10 adult (approximately 10 days) in the present chronic toxicity assay system. For the long-term exposure assay, 5-fluoro-2’-deoxyuridine (5-FUDR), an inhibitor of DNA synthesis used to prevent production of offsprings, was added to the treatment solution to get a final concentration of 25 μM (Wu et al., 2012a). All the exposures were carried out in incubators at 20 °C under dark conditions in the presence of food. During the long-term exposure, the exposed nematodes were transferred daily to a new petri dish containing fresh treatment solutions. Nematodes were fed UVC-killed *E. coli* to eliminate the potential confounding effects of bacterial metabolism (Meyer et al., 2010). After exposure, the exposed nematodes were washed three times with K medium, to prepare for the toxicity evaluation. Nematode growth, locomotion behavior, lifespan, lipofuscin accumulation, and stress-related genes expressions were chosen as the response endpoints. In addition, the population size of nematodes and stress response in TJ375 were also evaluated.

### 2.2. Evaluation of physiological indicators

Growth was investigated by body length. Before observation, the exposed nematodes were killed by heat, and then the length was measured by a microscope equipped with a graduated eyepiece (40 worms/treatment, 10/replicate). Locomotion behaviors were evaluated by head thrashes and body bends, and the assay was conducted as previously described (Wu et al., 2013). In each behavioral analysis, forty nematodes per treatment (10/replicate) were examined. Lifespan was evaluated by the mean value of all exposed nematode's lifespan in the same treatment group. Each nematode's lifespan was measured from egg to the time of death (30/replicate).

### 2.3. Evaluation of lipofuscin accumulation

Lipofuscin accumulation was tested using a fluorescence microscope (Nikon Eclipse 80i, UV-2A filter set) to take pictures. Intestinal autofluorescence was quantified using Adobe Photoshop.
CS3 (ver. 10.0) software by determining the average pixel intensity of nematodes’ intestine, and the background fluorescence was subtracted from the image data. Forty nematodes per concentration were examined.

2.4. RNA extraction and quantitative real-time PCR

In each treatment group, the exposed C. elegans from four replicates were harvested for RNA extraction. Experimental Procedure of RNA extraction and qRT-PCR analysis was conducted as previously described (Zhou et al., 2016a). RNA concentrations were determined by the absorbance at 260 nm. The quality of total RNA was estimated based on the ratio of the optical densities from RNA samples measured at 260 and 280 nm. The first-strand cDNA synthesis reaction was initiated with 500 ng of purified RNA by using FastQuant RT Kit (with gDNAse) (TIANGEN, China) according to the manufacturer’s protocol. The primers of 16 selected genes were shown in Table S1. Data was analyzed using the 2−ΔΔCt method as previously reported (Xu et al., 2013) and actin mRNA was used for the expression level normalization. For each tested gene, qRT-PCR analysis was conducted in triplicate (technical replicates).

2.5. Detection of population size of C. elegans

The experiment was conducted in 500-mL conical flasks containing 100 mL of treatment solutions with different BPA concentrations. One worm in the L4 stage from an age-synchronized culture was transferred into each test medium. The flasks were placed in the incubator shaker with a speed of 60 rpm/min at 20 °C, and bacterial food was added every 24 h. From the fifth day, 500 μL of the solution from each flask were taken each day to determine the total numbers of nematodes in each treatment group, by observing the nematodes under an optical microscope. The entire trial was independently repeated four times.

2.6. Detection of fluorescence signals from transgenic strain TJ375

After exposure, the analysis of transgenic nematode was conducted as previously described (Roh et al., 2006). Fluorescent images were observed using a fluorescence microscope (Nikon Eclipse 80i, FITC filter set) and the fluorescence intensities were quantified using a GENios microplate reader (TECAN, Switzerland), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Each tested well contained 50 nematodes of 4 replicates.

2.7. Statistical analyses

All data were expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using SPSS 12.0 (SPSS, Chicago, IL, USA). Analysis of variance (ANOVA) was used to determine the significance of differences between the groups. The Pearson correlation test was performed for the correlations between the parameters. The level for statistical significance was set as p < 0.05.

3. Results and discussion

3.1. Physiological effects of BPA on C. elegans

In the present study, C. elegans was exposed to low-concentration BPA from L4 larvae for 10 days. Following exposure, the survival rates of the exposed C. elegans showed no significant effects (data not shown). Growth showed significant (0.01–1 μM, p < 0.05; 10 μM, p < 0.01) decreases in the exposure groups from 0.01 μM to 10 μM (Fig. 1A; Raw data, Supplementary data, Table S2). Locomotion behaviors assayed by head thrashes and body bends were also evaluated (Fig. 1B and C). Exposure to BPA with concentrations above 0.001 μM significantly (p < 0.01) decreased the head thrashes of the exposed nematodes. As for the body bends, there were significant (p < 0.01) decreases in the exposure groups from 0.1 μM to 10 μM compared to the control. In addition, the mean lifespan of the exposed C. elegans was investigated as well. Significant (0.01 and 0.1 μM, p < 0.05; 1 and 10 μM, p < 0.01) decreases were observed in the exposure groups from 0.01 μM to 10 μM (Fig. 1D). With regard to the tested physiological indicators, the exposure group of 10 μM all showed the most negative effects.

The results indicated that head thrash was most sensitive among the tested endpoints for assessing the chronic toxicity of BPA, and the detection limit was as low as 0.001 μM (approximately 0.228 μg/L). Compared to the minimum concentration (0.01 μM) of toxic effects by acute and prolonged exposures (Zhou et al., 2016b), the long-term exposure could induce adverse effects on the physiological traits at a lower concentration. The non-effect doses of BPA in the short-term toxicity tests were still toxic to nematodes over the long term. Thus the concentrations of BPA lower than 0.228 μg/L should be safe for nematodes in the ecosystems. However, Jun et al. (2006) investigated the content levels of BPA in the surface waters of the Pearl River Estuary, Hengmen River and fishponds, and found that the BPA content in the three waters was 1.19, 1.41, and 2.06 μg/L, respectively. Masoner et al. (2014) studied fresh leachate from 19 landfills in the United States, and the results indicated that the mean and maximum BPA concentrations could reach 45.4 and 6380 μg/L. Considering the safety concentrations for C. elegans, environmental risks of BPA should receive more attentions. Nematodes, which play a key role in decomposition, nutrient cycling, and maintaining the environmental quality, are the most abundant metazoans in soil with a critical function in food webs. Any toxicity in the organism’s fitness could exert direct effects on the food webs, and even the whole ecosystems.

3.2. Biochemical effects of BPA on C. elegans

The effects at the biochemical level were also detected to evaluate the chronic toxicity of BPA. Similar to our previous study (Zhou et al., 2016b) using other assay systems (acute and prolonged exposures), there was also no significant difference in the lipofuscin accumulation between the exposure groups and the control (Supplementary data Fig. S1). Lipofuscin is a valuable aging marker of cellular damage, and the cellular damage is caused by the oxidative stress. In the present study, lipofuscin accumulation showed no significant effects, indicating oxidative stress, expressed by lipofuscin accumulation, was not induced at the exposure concentrations. Combined with our previous evaluation by acute and prolonged exposures (Zhou et al., 2016b), BPA exposure would not induce significant oxidative stress in C. elegans at the tested concentrations.

3.3. Molecular effects of BPA on C. elegans

The mRNA levels of 16 selected stress-related genes were detected to understand the molecular mechanisms of stress response in vivo. The selected genes encoded the proteins belonging to different metabolic pathways, and covered various stress response and defense systems, such as stress proteins, monooxygenase enzymes and antioxidant enzymes. Fig. 2 shows that BPA exposure led to increases in the mRNA levels of most genes at the tested concentrations (Gene expression date, Supplementary data Table S3). The degree of variation relative to the control was less in vit-2 (1.21–2.82 fold), vit-6 (1.44–2.83 fold), cyp-35a2 (1.06–1.67 fold), daf-12 (0.79–3.32 fold), age-1 (1.22–3.51 fold),
ape-1 (0.93–1.86 fold), and mtl-1 (0.96–2.28 fold), whereas the expressions of hsp-16.1, hsp-16.2, sod-1, sod-3, ctl-2, and cep-1 were...
strongly induced in response to the long-term exposure, and the variations were respectively 3.15–8.27 fold, 2.43–7.18 fold, 1.62–5.87 fold, 2.52–6.82 fold, 1.54–7.57 fold, and 3.21–9.86 fold compared to the control. Our previous study also evaluated the molecular effects of BPA by prolonged exposure (Zhou et al., 2016b). Compared to the prolonged exposure, the long-term exposure induced more obvious increases in the tested genes expressions at the same exposure concentration. From Fig. 2, the overall variations of gene expressions by BPA exposure were evidenced, and the variations could directly show the stress reaction in vivo. Combined with the physiological effects, the stronger stress response induced by the long-term exposure might be due to cumulative toxic effects.

3.4. Adverse effects of BPA on the increases in population size of C. elegans

Besides the effects at the individual level, the population-level effects of BPA exposure were also evaluated in the present study. Compared to the control, the population size of the exposed C. elegans was significantly (0.1 μM, p < 0.05; 1 and 10 μM, p < 0.01) decreased at the treatment groups from 0.1 μM to 10 μM (Fig. 3). The decrease in the population size indicated that the reproduction and life cycle of the exposed C. elegans were negatively affected. Our earlier study proved that the brood size of the exposed C. elegans significantly decreased by BPA exposure, and the numbers of germ cell corpses per gonad arm were also increased (Zhou et al., 2016b). Allard and Colaiácovo (2010) also showed that BPA exposure significantly impaired oogenesis, resulting in elevated levels of sterility and embryonic lethality. Any adverse effects on the production of larvae would result in more negative consequences at the population level (Forbes and Calow, 2002), thus the population size of nematodes was significantly decreased in the treatment groups from 0.1 μM to 10 μM.

3.5. Stress response in the transgenic strain TJ375

Eventually, the possibility of using transgenic C. elegans as a biosensor to monitor environmental toxicity was tested. The transgenic nematode TJ375 with the GFP based reporter hsp-16.2 was employed to obtain a rapid perception of environmental stress. Transgenic nematodes with the reporter hsp-16.2 have been used to reflect stress responses in previous studies (Roh et al., 2007; Shen et al., 2009), as heat shock proteins are general stress-response proteins protecting individuals from injury. In addition, the present study has proven that the hsp-16.2 gene expression showed obvious increases at the tested BPA concentrations. However, only in the treatment groups of 1 and 10 μM, the fluorescence signals from the GFP transgenic lines showed a significant (p < 0.05) increase compared to the control after chronic exposure (Supplementary data Fig. S2). The 1 μM treatment groups showed the most adverse effects, and obvious stress responses were induced in the exposed nematodes. The results indicated that the transgenic nematode TJ375 was able to detect BPA toxicity in the environment with concentrations more than 1 μM. Compared to the detection limit of 0.001 μM with the head thrash as the endpoint, the minimum detection concentration of 1 μM was much higher. Thus the transgenic line TJ375 seemed not sensitive towards BPA exposure, and a broad range of stress-related gene promoters to toxicants should be screened to identify the sensitive biosensor for toxicity evaluation.

3.6. Pearson correlation analyses of the investigated indicators

To show the correlations among the tested endpoints, Pearson correlation analyses were conducted on the 23 studied parameters (Supplemental Data Table S4). After the chronic exposure, 49 statistically significant correlations were detected. The results showed that parts of the significant correlations were found among the physiological endpoints and the population size. Other significant correlations were mostly found among the selected stress-related genes. Particularly, the gene expression of cep-1 was statistically associated with head thrash, body bend, stress responses in TJ375, and the gene age-1. Moreover, cep-1 is the only tested gene statistically associated with the physiological effects in the present study. Thus we speculated that the gene cep-1 might act as an important role in BPA-induced toxicity on C. elegans by the long-term exposure. Previous studies proved that the gene cep-1, required for DNA damage-induced apoptosis, regulated multiple stress responses in the soma, and mediated apoptosis and meiotic chromosome segregation in the germ line (Derry et al., 2001; Schumacher et al., 2001). Arum and Johnson (2007) also demonstrated that cep-1 expression was inversely associated with the lifespan of C. elegans. The Pearson correlation tests could show the potential relationships between the observed toxicity and specific genes, but linking molecular responses with ecotoxicological effects is very important and challenging in ecotoxicology. The statistical analyses only indicated a correlation, and the statistical relations could not reveal a causal relationship. Therefore, the biological meanings of the significant correlations between parameters should be further explained.

4. Conclusion

To conclude, long-term exposure to BPA could exert significant adverse effects on C. elegans at the physiological, molecular and population level. With the endpoint of head thrash, BPA exposure could have toxic effects at concentrations as low as 0.228 μg/L. The long-term exposure induced a more obvious stress response in vivo, and the gene cep-1 was speculated to act as an important role in BPA-induced toxicity on C. elegans by the chronic exposure. The chronic toxicity data could help accurately assess the environmental risks of BPA to ecosystems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.04.011.

References


