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Tetrachlorobisphenol A mediates reproductive toxicity in *Caenorhabditis elegans* via DNA damage-induced apoptosis

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HIGHLIGHTS

- TCBPA exposure induced severe reproductive toxicity via germline apoptosis.
- TCBPA at 100 μg/L regulated the expression of the germline apoptosis pathway.
- DNA damage was observed following *hus-1::GFP* exposure to TCBPA.
- TCBPA-mediated reproductive toxicity occurred via DNA damage-induced apoptosis.

GRAPHICAL ABSTRACT

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ABSTRACT

Tetrachlorobisphenol A (TCBPA), an alternative to tetrabromobisphenol A (TBBPA), is ubiquitous in the environment and could potentially impact the reproductive system of organisms. However, the mechanisms underlying TCBPA-mediated reproductive effects remain unclear. Herein, we exposed *Caenorhabditis elegans* (*C. elegans*, L4 larvae) to TCBPA at environmentally relevant doses (0–100 μg/L) for 24 h. Exposure to TCBPA at concentrations of 1–100 μg/L impaired fertility of *C. elegans*, as indicated by brood size. After staining, the number of germline cells decreased in a dose-dependent manner, whereas germline cell corpses increased in exposed nematodes (10–100 μg/L TCBPA). Moreover, the expression of genes related to the germline apoptosis pathway was regulated following exposure to 100 μg/L TCBPA, indicating the potential role of DNA damage in TCBPA-induced apoptosis. Apoptosis was nearly abolished in *ced-4* and *ced-3* mutants and blocked in *hus-1*, *egl-1*, *cep-1*, and *ced-9* mutants. Numerous foci were detected in TCBPA (100 μg/L)-exposed *hus-1::GFP* strains. These results indicate that TCBPA induces *hus-1*-mediated DNA damage and further causes apoptosis via a *cep-1*-dependent pathway. Our data provide evidence that TCBPA causes reproductive toxicity via DNA damage-induced apoptosis.

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1. Introduction

Tetrabromobisphenol A (TBBPA), one of the most important brominated flame retardants (BFRs), is widely used in plastics, textiles, electronic devices, and construction materials (Stapleton et al., 2005; Li et al., 2019). Owing to its high flammability, TBBPA accounts for the largest proportion of BFRs (Jarosiewicz and Bukowska, 2017). However, its widespread utilization facilitates the release of TBBPA into different environmental matrixes via physical processes such as volatilization, lixiviation, and abrasion, further resulting in environmental and biological damage (Fijalkowski et al., 2017). As an alternative to TBBPA, tetrachlorobisphenol A (TCBPA), which has a similar chemical structure (Supplementary Data, Fig. S1), has been widely used in recent decades. Studies have reported that TCBPA can be detected in river sediments and sludges at concentrations ranging from 142.5 to 542.6 ng/g dw (Yuan et al., 2010; Song et al., 2014b). The average TCBPA level in river water was found to be 224 ng/L, whereas that of TBBPA was 143 ng/L (Yin et al., 2011). Owing to its lipophilicity, TCBPA has been measured in various organisms, including the human body. For example, the TCBPA level was 0.143 ng/mL in the serum of pregnant women (Li et al., 2020). It should be noted that TCBPA might exert more severe toxicity than TBBPA; thus, the risks associated with TCBPA exposure are also concerning (Zhang et al., 2018).

Ecotoxicological studies have reported that TCBPA exhibits multiple adverse effects, such as interference with the endocrine system, reproductive toxicity, and cytotoxicity (Kitamura et al., 2005; Yin et al., 2018b). TCBPA can reportedly disrupt thyroid hormone levels, influence embryonic development, and induce developmental toxicity in rats (Sun et al., 2009). Similarly, TCBPA exposure can inhibit development and reproduction in *Oryzias melastigma* (Huang et al., 2017). In addition, TCBPA can alter locomotive behaviors and disrupt the neuronal transmission process, which may cause developmental neurotoxicity (Zatecka et al., 2013; Liang et al., 2019). Relevant studies assessing TCBPA toxicity are still in the early stages, and limited knowledge is available regarding the mechanisms underlying TCBPA. Given the importance of TBBPA, the potential mechanisms of action must be clarified.

Caenorhabditis elegans (*C. elegans*) is an excellent model animal owing to its low cost, short life cycle, large-scale offspring, and high sensitivity to pollutants, which can be used to evaluate the toxicity of environmental pollutants, including persistent organic pollutants, materials, and metals (Wang et al., 2018; Kim et al., 2019; Moon et al., 2019; Zhang and Yu, 2020). In addition, *C. elegans* can be employed to investigate the potential molecular mechanisms of chemicals, given its high homology with the human genome (up to 80%) (Kaletta and Hengartner, 2006). In the present study, *C. elegans* was used to dissect the potential reproductive toxicity of TCBPA at environmentally relevant concentrations, with an emphasis on apoptosis. The results would provide a theoretical basis for the health risk assessment of TCBPA and will be valuable for establishing relevant regulations.

2. Materials and methods

2.1. C. elegans strain and culture conditions

Wild-type N2, mutants, and transgenic strains were used in this study, including WS2277 *hus-1* (*op241*), MT1082 *egl-1* (*n487*), JR1279 *cep-1* (*w40*), MT4770 *ced-9* (*n1950*), MT2547 *ced-4* (*n1162*), and MT1522 *ced-3* (*n717*). The transgenic strain was WS1433 *hus-1::GFP* (*opIs 34*). All strains were obtained from the Caenorhabditis Genetics Center and cultured on nematode growth media (NGM) plates with *Escherichia coli* OP50, as previously described (Brenner, 1974). The nematodes were maintained in a sterile environment at a constant temperature (20 °C). To meet requirements for synchronous development, eggs were released from gravid hermaphrodites using lysis buffer and incubated for 48 h to acquire L4-larvae nematodes for subsequent experiments.

2.2. TCBPA exposure procedures

TCBPA (CAS NO: 79-95-8) was purchased from Aladdin Corporation (Shanghai, China). A stock solution (1 mg/mL) was prepared using dimethyl sulfoxide (DMSO), followed by dilution with K medium to obtain different exposure solutions. Based on concentrations detected in the environment (Fukazawa et al., 2001; Yin et al., 2011), nematodes were subjected to TCBPA exposure at environmentally relevant concentrations of 0.01, 0.1, 1, 10, and 100 μg/L. As a vehicle control, *C. elegans* was treated with 0.01% DMSO. The control and treated nematodes were exposed to 6-well plates for 24 h. Independent experiments were performed in quadruplicate.

2.3. Lethality and fertility ability assay

Lethality was assayed by counting the total number of live nematodes during the acute exposure process. Fertility was evaluated by assessing the brood size as the endpoint. After acute exposure to TCBPA at 0–100 μg/L, a single nematode from each concentration was randomly selected and placed onto new a NGM plate with an OP50 lawn daily. Brood size was counted using a stereomicroscope. At least 10 nematodes were assayed per concentration, and independent experiments were performed in quadruplicate.

2.4. Measurement of germline apoptosis

4′,6-Diamidino-2-phenylindole (DAPI) is a well-known dye that penetrates the cell membrane and strongly binds to DNA, emitting a bright blue fluorescence. DAPI was used to stain germline cells in *C. elegans*. After exposure, nematodes were washed three times with K medium to remove excreta. Subsequently, nematodes were transferred to glass slides with levamisole for 30 min (Chen et al., 2019). Nematodes were stained with DAPI (0.2 μg/mL) for 10 min. The results were obtained by analyzing fluorescent images. Forty nematodes were examined at each concentration.

Acridine orange (AO) staining is a crucial method for measuring apoptotic germline cells, as previously reported (Wang et al., 2014a). After acute exposure, 40 nematodes per concentration were stained with AO solution, provided OP50 lawn, and incubated at 20 °C for 60 min under dark conditions. The nematodes were washed with K medium and immobilized with levamisole for 30 min. Fluorescent images were captured using an Olympus BX51 microscope, and the apoptotic germline cells were counted.

2.5. Measurement of DNA damage

Using the transgenic strain WS1433, DNA damage was evaluated based on the number of bright foci in the nuclei of germline cells. After acute exposure to TCBPA at 0 and 100 μg/L, the nematodes were anesthetized on agar plates for 30 min. Images were captured using a fluorescence microscope (Olympus BX51, Japan). Fifty nematodes were examined at each concentration.

2.6. Quantitative real-time PCR (qRT-PCR) analysis

Nematodes exposed to 0 and 100 μg/L TCBPA were removed and

washed with K medium several times in enzyme-free centrifuge tubes. Highly purified RNA was obtained using the RNAsimple Total RNA Kit (Tiangen, China). A Veriti 96-well Thermal cycler (Applied Biosystems, USA) was used to synthesize cDNA from RNA via reverse transcription. The expression of genes related to germline apoptosis was measured using SYBR Green RT-qPCR master mix on an Applied Biosystems StepOnePlus Real-Time PCR System (Thermo Fisher Scientific, USA). PCR was performed at 95 °C for 30 s, 40 cycles at 95 °C for 5 s, and 60 °C for 30 s. The results are presented as relative expression ratios. All reactions were performed in triplicate. The designed primers are presented in the Supplementary Data (Table S1).

2.7. Statistical analyses

Data are expressed as mean ± standard error of the mean (SEM). Graphs were created using Origin 8.0. To explore the significance of differences between concentrations, a one-way analysis of variance (ANOVA) with Tukey's test was performed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). Differences at $p < 0.05$ and $p < 0.01$ were considered statistically significant.

3. Results

3.1. Effects of TCBPA on fertility

Lethality and brood size were examined in *C. elegans* after exposure to TCBPA for 24 h at environmentally relevant concentrations. Acute exposure to TCBPA at doses of 0.01–100 μg/L did not impact nematode survival (Supplementary Data, Fig. S2). Moreover, the number of offspring was not notably affected by TCBPA exposure (0–0.1 μg/L) (Fig. 1). However, higher concentrations (≥1 μg/L) of TCBPA significantly reduced brood size. Compared with the control group, exposure to 100 μg/L TCBPA reduced the number of offspring by 26.80%.

3.2. Effects of TCBPA on apoptosis

Based on the reproductive effects of TCBPA in exposed nematodes, we speculated that TCBPA might induce germline cell apoptosis in *C. elegans*. After TCBPA exposure for 24 h, we performed DAPI and AO staining. Germline cells appeared blue after DAPI staining, whereas apoptotic cells appeared yellow-green after AO staining. As shown in Fig. 2A, exposure to low concentrations (0.01 and 0.1 μg/L) of TCBPA exerted no adverse effect on germline cells when compared with the control group. However, a marked decrease in the number of germline cells was observed, and the effects were statistically significant at concentrations of 1–100 μg/L. Following exposure to 100 μg/L TCBPA, germline apoptosis was detected, and the number of germline cells decreased by almost 50% compared with the control group. Consistent with these results, apoptotic germline cells were observed in exposed nematodes after AO staining, and acute exposure to 10–100 μg/L TCBPA induced death in more germline cells (Fig. 2B). Considering that 100 μg/L resulted in more severe toxic effects, this concentration was used to explore the mechanisms of action of TCBPA in *C. elegans*.

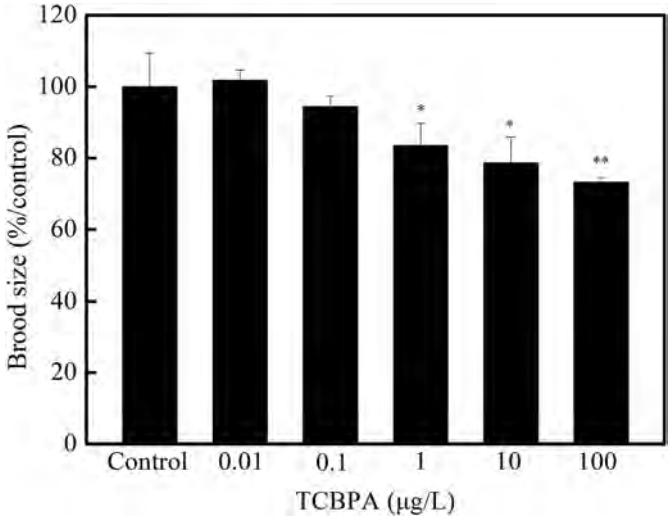

Fig. 1. Effect of TCBPA on nematode fertility after acute exposure. Comparison of brood size of *C. elegans*. Data (mean ± SEM) are expressed as the percentage value compared with the control group. The asterisks indicate significant differences between exposure and control groups. *$p < 0.05$, **$p < 0.01$.

3.3. Effects of TCBPA on the germline apoptosis pathway

To comprehensively elucidate the molecular mechanisms of TCBPA, genes involved in the germline apoptosis pathway (*ced-3*, *ced-4*, *ced-9*, *efl-2*, *egl-1*, *egl-38*, *cep-1*, *dpl-1*, *efl-1*, *lin-35*, *sir-2.1*, and *pax-2*) were selected for qRT-PCR. The *tba-1* gene, encoding the alpha-tubulin protein, was used to normalize the fold-change in the exposure group (100 μg/L). Compared with the control group, exposure to 100 μg/L TCBPA significantly upregulated expression levels of *ced-3*, *ced-4*, *egl-1*, *egl-38*, *cep-1*, *efl-2*, *efl-1*, *lin-35*, and *sir-2.1*, with expression changes varying from 1.22- (*ced-4*) to 3.51-fold (*egl-1*) (Fig. 3). In contrast, the expression of *ced-9* and *pax-2* was significantly downregulated by 0.60- and 0.63-fold, respectively. Overall, these findings indicated that TCBPA exposure induces a genotoxic response in the germline apoptosis pathway.

3.4. Effects of TCBPA on DNA damage mechanism

Cep-1, a member of the p53 family, is mainly associated with DNA damage-induced apoptosis (Baruah et al., 2014). In terms of gene expression, we examined DNA damage related to germline apoptosis. *Hus-1*, a nuclear checkpoint protein, can be relocalized to distinct foci of double-strand breaks after DNA damage (Hofmann et al., 2002). Higher nematode relocation was observed in the 100 μg/L TCBPA-exposed *hus-1:: GFP* strain than in the control group (Fig. 4). Brighter foci were notably observed under the microscope, indicating that TCBPA induces severe DNA damage in nematodes.

3.5. Role of core apoptotic machinery in germline apoptosis

To confirm the role of these genes in TCBPA-induced germline apoptosis, we employed *hus-1* (*op241*), *egl-1* (*n497*), *cep-1* (*w40*), *ced-9* (*n1950*), *ced-4* (*n1162*), and *ced-3* (*n717*) mutants. In all mutants, exposure to TCBPA at the highest concentration (100 μg/L) did not significantly affect apoptosis when compared with the control group (Fig. 5). After exposure to *ced-3* (*n717*) and *ced-4* (*n1162*) strains, almost no germline cell corpses were detected in control and 100 μg/L TCBPA-exposed groups. Furthermore, compared with N2 wild-type worms, *ced-9* (*n1950*), *cep-1* (*w40*), *egl-1* (*n497*), and *hus-1* (*op241*) strains showed a slight increase in the number of germline cell corpses under TCBPA-free conditions, whereas fewer apoptotic cells were noted at 100 μg/L TCBPA. This genetic evidence further demonstrates that genes involved in DNA damage contribute to TCBPA-induced apoptosis by activating the anti-apoptotic regulator *ced-9*.

4. Discussion

Halogenated BPA analogs, such as TBBPA and TCBPA, are used as flame retardants in packaging materials for foods, automobile parts, and other products (Summerfield et al., 1998). Given their high production volumes and wide applications, halogenated bisphenol A analogs can be frequently are widely detected in the environment and various organisms. For example, the concentration of TBBPA was found to reach up to

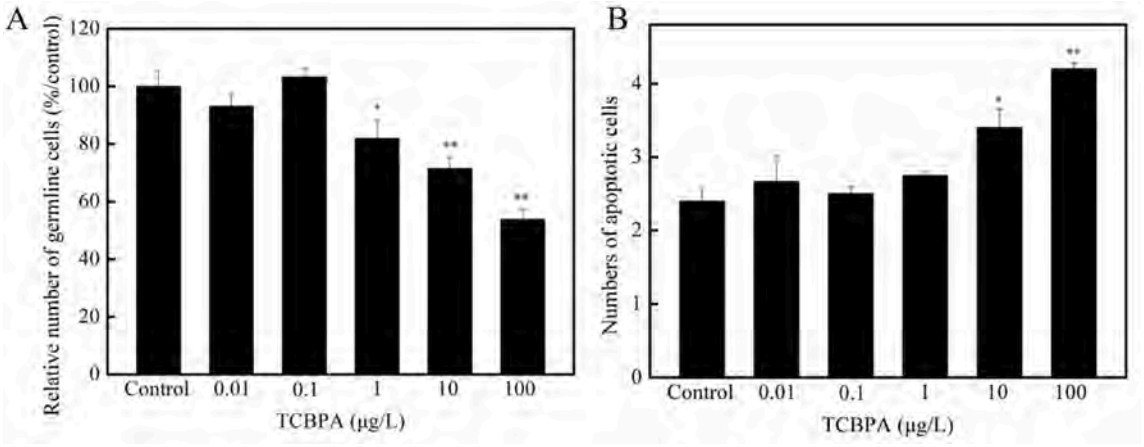

Fig. 2. Effect of TCBPA on cell apoptosis in nematodes after acute exposure. (A) Relative number of germline cells after DAPI staining; (B) Numbers of apoptotic cells after AO staining. The asterisks indicate significant differences between the exposure and control groups. *$p < 0.05$, **$p < 0.01$.

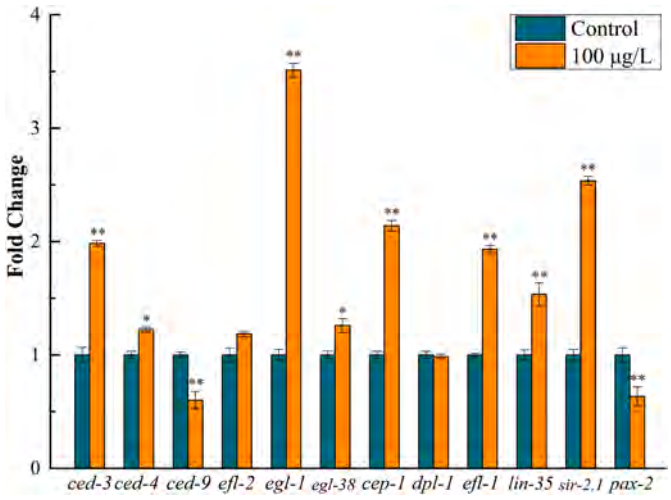

Fig. 3. Gene expression of the germline apoptosis pathway after acute exposure to 100 μg/L TCBPA. Values of gene expressions are normalized using *tba*-1 mRNA and presented as means (n = 3) relative to the control. *$p < 0.05$.

4870 ng/L in Chaohu Lake (Liu et al., 2016), 2 μg/L in the treated water from paper mills (Fukazawa et al., 2001), and 7.7 ng/L in drinking water (Fan et al., 2013). Considering exposure pathways, halogenated BPA analogs can harm to organisms and human health through diet, dermal contact, and dust inhalation (Abdallah et al., 2015). Previous studies have shown that halogenated BPA analogs cause developmental and reproductive toxicity in multiple species, and some recent studies have reported that TCBPA is more toxic than TBBPA (Kuiper et al., 2007a; Song et al., 2014a; Zatecka et al., 2014; Linhartova et al., 2015). Therefore, in the present study, we investigated TCBPA-mediated toxicity at environmentally relevant concentrations in *C. elegans*.

Although exposure to 0.75 mg/L TCBPA resulted in a 50% mortality rate in zebrafish larvae (Song et al., 2014a), acute exposure to TCBPA did not influence the survival of nematodes in the present study. Similarly, subacute exposure to TBBPA at concentrations of 0–200 μg/L did not impact survival in *C. elegans* (Liu et al., 2020). Reproduction is substantially vulnerable to harmful chemicals, as determined by examining brood size (Dubois et al., 2019). The reduction of daily and total brood size was detected in 1.08 mg/L TBBPA-exposed nematodes by utilizing the L4 larvae of *C. elegans* (Saul et al., 2014). TCBPA at 0.2 mg/L reportedly reduced the hatching rate and delayed hatching time in *Oryzias melastigma* (Huang et al., 2017). However, we detected a difference in the nematode sensitivity to TCBPA exposure. After acute exposure, TCBPA at concentrations ≥1 μg/L decreased brood size, indicating that TCBPA had a more pronounced toxic effect on reproduction than TBBPA. Typically, these results suggest that TCBPA at environmentally relevant concentrations has no lethal effect but may cause reproductive toxicity and affect egg-laying behavior in *C. elegans*.

Apoptosis, a programmed cell death process, resists interference from external stimuli and maintains homeostasis in organisms. The production of germline cell corpses in targeted reproductive organs is markedly activated in response to environmental pollutants during the oogenesis (Salinas et al., 2006; Cheng et al., 2014; Wang et al., 2014b). Compared with the control group, TCBPA at 1 μg/L significantly increased germline apoptosis in the gonads of *C. elegans*, as determined by AO staining, whereas the number of germline cells was significantly decreased after DAPI staining. Previous studies have reported that TBBPA exposure causes testicular cell apoptosis, resulting in a reduction in normal sperm and reproductive toxicity (Kuiper et al., 2007b; Zatecka et al., 2013; Linhartova et al., 2015). Furthermore, the number of germline cells was significantly decreased when compared with that in the pristine nanopolystyrene group, along with germline cell corpses detected following exposure to amino-modified nanopolystyrene at 10 μg/L, thereby contributing to reproductive toxicity (Qu et al., 2019). These results provide direct evidence of imply a close relationship between apoptosis and reproductive toxicity induced by TCBPA at environmental concentrations.

In addition, we examined potential molecular mechanisms using the observed germline cell apoptosis. In *C. elegans*, *ced-3* and *ced-4* are cell death abnormality genes that promote apoptosis; however, *ced-9* is an anti-apoptotic factor that protects protect cells against programmed death during nematode development (Pourkarimi et al., 2012; Bailly and Gartner, 2013). Lipopolysaccharide (LPS) exposure reportedly increases *ced-3* but decreases *ced-9* expression, suggesting that LPS induces apoptosis (Ma et al., 2020). Likewise, our findings revealed that exposure to 100 μg/L TCBPA upregulated *ced-3* expression by 1.98-fold and downregulated *ced-9* gene expression by 0.60-fold. Moreover, the expression of *cep-1* was significantly upregulated, which promoted the combination of *egl-1* and *ced-9* and induced the core apoptotic pathway. *Egl-1*, which encodes a BH3-only protein, is an upstream activator of the core apoptotic pathway and plays a key role in mitochondria (Nehme and Conradt, 2008). *Cep-1*, encoding a p53-like protein, responds to DNA damage checkpoints and induces apoptosis via positive regulation of *egl-1* (Lettre and Hengartner, 2006). Previous studies have shown that environmental pollutants, such as TBBPA and BPA, can significantly increase the expression of *cep-1* and *egl-1* (Yin et al., 2018a; Liu et al., 2019). Another study has reported that chronic exposure to 100 nM methylmercury (MeHg) increased the expression of *ced-3*, *ced-4*, *cep-1*, and *egl-1* gene, indicating that these genes may be associated with

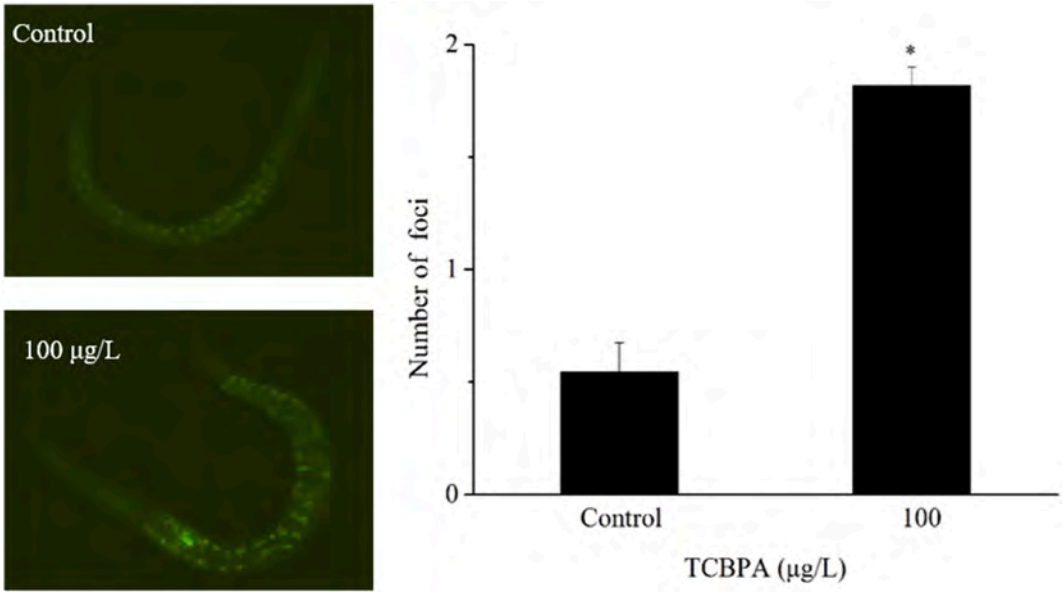

Fig. 4. DNA damage in *hus-1* transgenic strain after acute exposure to 100 μg/L TCBPA. Representative images and the number of foci per 50 germ cells in control and TCBPA groups. *$p < 0.05$.

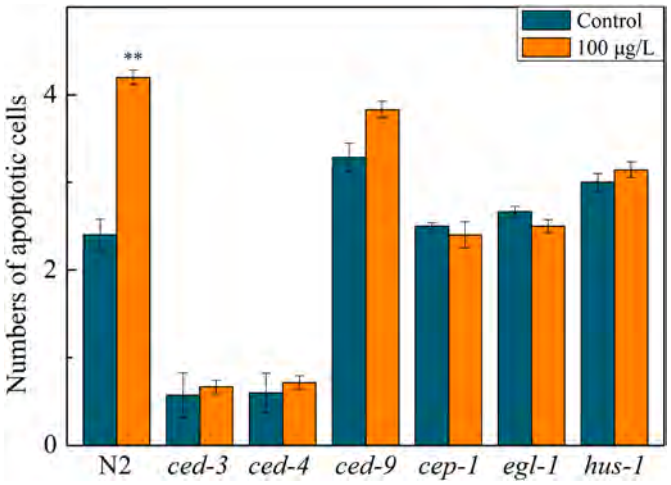

Fig. 5. Cell apoptosis in *ced-3* (*n717*), *ced-4* (*n1162*), *ced-9* (*n1950*), *cep-1* (*w40*), *egl-1* (*n497*) and *hus-1* (*op241*) mutants after acute exposure to 100 μg/L TCBPA. Numbers of apoptotic cells in control and TCBPA groups. **$p < 0.01$.

MeHg-induced reproductive toxicity (Hu et al., 2021). Therefore, TCBPA may induce reproductive toxicity via DNA damage-activated *cep-1*-dependent apoptosis, where *ced-3*, *ced-9*, *cep-1*, and *egl-1* play critical roles.

Germline apoptosis is activated by DNA damage via the mitochondrial pathway (Stergiou and Hengartner, 2004). For example, exposure to endosulfan at 1 μM can significantly increase the number of *hus-1::GFP* foci, indicating that endosulfan causes reproductive toxicity via DNA damage (Du et al., 2015). Our results revealed that 100 μg/L TCBPA could induce DNA damage, suggesting that DNA damage contributes to TCBPA-mediated reproductive toxicity. Furthermore, low levels of apoptosis were observed in ced-3 (n717) and ced-4 (n1162) strains, and other TCBPA-exposed strains showed marginal changes in the number of germline cell corpses, thereby indicating that apoptosis was blocked in these mutant nematodes. These results illustrate that DNA damage mediated by hus-1 triggers the cep-1 pathway and induces the germline cell apoptosis. Similarly, mutations in *C. elegans* ced-3 and ced-4 block germline cell death when exposed to environmental pollutants such as copper and tributyltin (Wang et al., 2009, 2014b). Conversely, the number of apoptotic germline cells reportedly increased in a dose-dependent manner in hus-1, egl-1, and cep-1 mutants after BDE-47 exposure (3–30 μg/mL) for 24 h, suggesting that the cep-1-dependent pathway is not involved in BDE-47-mediated reproductive toxicity (You et al., 2018). Based on the findings of the present study, DNA damage can be deemed critical for inducing TCBPA-mediated apoptosis and reproductive toxicity.

5. Conclusion

Exposure to TCBPA at environmentally relevant concentrations led to reproductive toxicity and accelerated germline apoptosis. Analysis of the germline apoptosis pathway revealed that TCBPA-induced apoptosis may be caused by DNA damage and is mediated by a *cep-1*-dependent pathway in the mitochondria. Moreover, the underlying molecular mechanism was found to be associated with TCBPA-induced DNA damage, mediated by *hus-1*, which causes apoptosis via a *cep-1*-dependent pathway. These results improve our understanding of TCBPA-mediated reproductive toxicity and its underlying mechanisms.

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 paper.

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Appendix A. Supplementary data

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