Effect of Tri-n-butyl phosphate (TnBP) on neurobehavior of Caenorhabditis elegans

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Abstract

As an emerging flame retardant, organic phosphate flame retardants have been extensively used worldwide. The aim of this study is to determine the effects of TnBP on neurobehavior of Caenorhabditis elegans (C. elegans) and its mechanisms. L1 larvae of wild-type C. elegans N2 and transgenic nematodes (BZ555, DA1240 and EG1285) were exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours, respectively. Then, we observed that TnBP inhibited the body length and body width, increased the head swings and body bends, reduced the pump contraction times and chemical trend index, and increased the production of reactive oxygen species (ROS), altered the expression of genes (mev-1 and gas-1) related to mitochondrial oxidative stress, increased the expression of genes (pmk-1, sek-1 and nsy-1) related to p38 MAPK signal pathway, increased the production of dopamine, glutamate and Gamma-Amino Butyric Acid (GABA). When changes of motor behaviors were taken as the endpoint of toxicity evaluation, we found that the pmk-1 mutants led to the sensitivity of nematodes to TnBP. Therefore, the results showed that TnBP had harmful effects on the neurobehavior of nematodes, oxidative stress might be one of the mechanisms of neurotoxicity, and the activation of p38 MAPK signal pathway might play a protective role for nematodes against the harmful effects induced by TnBP. The research results revealed the potential effects of TnBP on the neurobehavior of C. elegans, put forward new expectations for the study of environmental hygiene and human survival risks of organic phosphate flame retardants.

1. Preface

Polybrominated diphenyl ether was once one of the most common flame retardant chemicals, which was widely added to various consumer goods, such as textiles, furniture, electronic products, etc., to delay ignition time and slow down combustion rate(Percy, Z, Vuong, A M and Ospina, M, et al., 2020). However, halogenated flame retardants have the characteristics of long-distance migration, persistence, bioconcentration and ecotoxicity, and have the effects of neurological, immune toxicity and reproductive toxicity, endocrine interfere, and other health damage to people. At present, halogenated flame retardants have been banned all over the world. The organophosphate esters (OPEs) include a large class of chemical substances with similar structures, that is $S = P(OR)_3$ or $O = P(OR)_3$, where “R” represents different aromatic and aliphatic groups, which can be connected to the main chain of the molecule(van der Veen, I and de Boer, J, 2012). With the prohibition of brominated flame retardants, organic phosphate flame retardants have become their substitutes and have been widely used all over the world(Blum, A, Behl, M and Birnbaum, L S, et al., 2019). In addition to having been used as a flame retardant, OPEs also have been widely used as pesticides, herbicides, plasticizers and other additives to various consumer goods, such as plastics, furniture, textiles, construction, automotive and petroleum industries(THOMPSON, C M, PRINS, J M and GEORGE, K M, 2010; van der Veen, I and de Boer, J, 2012). OPEs include TnBP, tri-iso-butyl phosphate (TiBP), tris (2-ethylhexyl) phosphate (TEHP), tris (2-chloroethyl) phosphate (TCEP), tris-2-butoxyethyl phosphate (TBOEP), tris (chlorisopropyl) phosphate (TCPP), and so on(Li, J, Zhao, L and Letcher, R J, et al., 2019). Most organic phosphate flame retardants are added to all kinds of consumption goods only by physical means rather than chemical bonding(Meeker, J D, Cooper, E
M and Stapleton, H M, et al., 2013; Adibi, J J, Perera, F P and Jedrychowski, W, et al., 2003). Therefore, OPEs and the products containing OPEs are very easy to enter the various environment media through volatilization, product wear and leakage in the process of production, transportation, use, waste, and disposal. Studies indicated that OPEs were extensively detected from a variety of environmental media such as air, dust, water, sediment, soil as well as organisms. The $\Sigma$OPEs (the total concentration of OPEs) was almost 0.005 ng/m$^3$ $\sim$ 576.73 ng/m$^3$ in air (indoor air and outdoor air) (Wang, X, Zhu, Q and Yan, X, et al., 2020). The $\Sigma$OPEs was tens to hundreds of ng/m$^3$ in indoor air, which was generally 10 $\sim$ 1000 times higher than those found in outdoor air (Wang, X, Zhu, Q, Yan, X, Wang, Y, Liao, C and Jiang, G, 2020). The $\Sigma$OPEs was reported to be 36.6 ng/L $\sim$ 32000 ng/L from various water environment in China (Wang, X, Zhu, Q, Yan, X, Wang, Y, Liao, C and Jiang, G, 2020). The mean concentration of OPEs in global water sediment was 0.88 ng/g $\sim$ 236 ng/g (Wang, X, Zhu, Q, Yan, X, Wang, Y, Liao, C and Jiang, G, 2020). In the sediments of Liaohe River in northern China, the total concentration of 13 OPEs was 19.7 ng/g $\sim$ 234 ng/g, and the mean concentration was 64.2 ng/g (Luo, Q, Gu, L and Wu, Z, et al., 2020), with the main pollutants including TiBP, TnBP and TBOEP. The $\Sigma$OPEs was 0.041 ng/g $\sim$ 14000 ng/g from various soil environments in China (Patisaul, H B, Behl, M and Birnbaum, L S, et al., 2021). In biota, $\Sigma$OPEs was 1.8 ng/g wet weight $\sim$ 22 ng/g wet weight (Ma, Y, Cui, K and Zeng, F, et al., 2013). OPEs can enter the human body from various environmental media through a variety of routes. Inhalation through respiratory tract was considered be the main exposure route of OPEs (especially volatile OPEs, such as TCPP and TCEP) (Schreder, E D, Uding, N and La Guardia, M J, 2016). The OPEs with low volatility mainly exist in dust, so it was mainly ingested into the body with dust (Cequier, E, Sakhi, A K and Marce, R M, et al., 2015; Larsson, K, de Wit, C A and Sellstrom, U, et al., 2018). In addition, when the semi-volatile OPEs were accumulated on hands due to being exposed to contaminated dust or contacting products containing OPEs with hand, they could enter the body through hand-mouth transfer, skin absorption, etc. (Hammel, S C, Hoffman, K and Lorenzo, A M, et al., 2017). Different OPEs have diverse toxic effects due to the distinct structures. Organophosphorus insecticides can strongly inhibit the activity of acetylcholinesterase (AChE), while organic phosphate flame retardants hardly affect AChE activity (Sun, L, Xu, W and Peng, T, et al., 2016). In fact, in 2019, the review committees proposed that organic phosphate flame retardants were at most weak inhibitors of AChE, and it seemed unlikely that they will produce neurotoxicity through other mechanisms such as aminobutyric acid inhibition at the current levels of human exposure (Patisaul, H B, Behl, M and Birnbaum, L S, et al., 2021). However, there was evidence that organic phosphate flame retardants could affect neural transmission pathways that are extremely important for brain development, thereby affecting neural development (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021). In addition, some OPEs can also be metabolized into more toxic compounds. Experimental animal studies showed that some OPEs had neurotoxicity, carcinogenicity, endocrine interfere and reproductive toxicity (Xu, Q, Wu, D and Dang, Y, et al., 2017; van der Veen, I and de Boer, J, 2012; Kojima, H, Takeuchi, S and Itoh, T, et al., 2013). For example, TPHP, TnBP, TBOEP, and TCEP were developmental neurotoxicants (Sun, L, Tan, H and Peng, T, et al., 2016). Tang et al. found that exposure to TDCPP of 0, 0.1, 1, 10, and 100 mg/L for 72 hours could affect neurobehavior of C. elegans, such as inhibiting the movement of nematodes and reducing the number of dopamine neurons, and the neural damage might
be bound up with oxidative stress as well as p38 MAPK signal pathway (Tang, J, Li, J and Zhou, Q, et al., 2022).

As a typical alkyl OPE, TnBP has been widely used as industrial defoamer, rare metal extractant and chlorinated rubber plasticizer (Garcia, M, Rodriguez, I and Cela, R, 2007). Meanwhile, TnBP is also a very common organic phosphate flame retardant. TnBP can persist in the environment media due to its stable nature, viscous properties and strong resistance for natural photolysis and hydrolysis (Zhang, H, Liu, T and Song, X, et al., 2021). The concentration of TnBP was 0.01 ng/m$^3$ ~ 190 ng/m$^3$ from various air environments in China (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021) and the median concentration of TnBP was 0.0011 ng/m$^3$ ~ 75 ng/m$^3$ in various indoor air (such as dwellings, elementary schools, houses, child care facilities, childcare centers, preschools, infant homes, etc.) from different microenvironments of many countries such as Sweden, Norway, Germany, Japan, US, Australia, etc. (Hou, M, Shi, Y and Na, G, et al., 2021). The concentration of TnBP was 0.09 ng/g ~ 5367 ng/g from various dust environments in China (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021) and the median concentration of TnBP was 0.0021 ug/g ~ 5.6 ug/g in various indoor dust (such as dwellings, elementary schools, houses, child care facilities, preschools, etc.) from different microenvironments of many countries such as Canada, New Zealand, Sweden, Spain, Germany, UK, Japan, US, USA, etc. (Hou, M, Shi, Y, Na, G and Cai, Y, 2021). The concentration of TnBP in various water environments in China was 0 ng/L ~ 1132 ng/L (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021). The concentration of TnBP was 0.1 ng/L ~ 80.9 ng/L and the mean concentration was 6.3 ng/L, among the 40 rivers which flow into the Bohai Sea in China (Wang, R, Tang, J and Xie, Z, et al., 2015). It was found that the concentration of TnBP in fish from the Pearl River Delta was 43.9 ng/g lw ~ 2946 ng/g lw (Ma, Y, Cui, K and Zeng, F, et al., 2013) in the south of China. The concentration of TnBP was 0 ng/g ~ 15332 ng/g from various sediment environments in China (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021). The concentration of TnBP was 0.002 ng/g ~ 112 ng/g from various soil environments in China (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021). The concentration of TnBP was 0.79 ng/g wet weight ~ 2946 ng/g wet weight in biota in China (Patisaul, H B, Behl, M, Birnbaum, L S, Blum, A, Diamond, M L, Rojello, F S, Hogberg, H T, Kwiatkowski, C F, Page, J D, Soehl, A and Stapleton, H M, 2021). In a maternal infant cohort study from Norway, TnBP was detected from each maternal infant sample (Kucharska, A, Cequier, E and Thomsen, C, et al., 2015). The widespread detection of TnBP from various environmental media and biological tissue samples has aroused people's extensive attention to whether TnBP will have harmful effects on the environmental hygiene and human survival. Therefore, the research on the toxic effect of TnBP has increased year after year. It was found that TnBP could affect reproductive function, interfere with endocrine and nervous system development, and might be carcinogenic. Chang et al. found that TnBP exposure could increase the activities of lactate dehydrogenase and Caspase-3 in PC12 cells, reduce cell viability and AchE activity, and change cell
morphology, suggesting the neurotoxicity of TnBP to PC12 cells (Chang, Y, Cui, H and Jiang, X, et al., 2020). TnBP might lead to neurotoxicity to PC12 cells mainly through inducing apoptosis and affect neuronal differentiation by inhibiting AChE activity (Chang, Y, Cui, H, Jiang, X and Li, M, 2020). Jiang et al. found that the activities of glutamate, Ca^{2+}, Na^{+}/K^{+}-ATPase and Ca^{2+}-ATPase were influenced by TnBP in earthworm brain ganglion when exposed to TnBP, indicating the neurotoxicity of TnBP to earthworms, and the main reasons for the neurotoxicity of TnBP to earthworms were osmotic imbalance and calcium overload (Jiang, X, Yang, Y and Liu, P, et al., 2020). Zhang et al. found that TnBP had reproductive toxicity to C. elegans, and based on this, it was predicted that TnBP also had reproductive toxicity to mammals (Zhang, H, Liu, T, Song, X, Zhou, Q, Tang, J, Sun, Q, Pu, Y, Yin, L and Zhang, J, 2021). The tertiary butyl phosphate (TBP) includes TnBP and TiBP, which have the same molecular formula (C_{12}H_{27}O_{4}P) and different structural formula, namely isomer. The structures of TnBP and TiBP were shown in Fig. 1 (a) and (b), respectively. Laham et al. found that TBP (0.42 mL/ kg·d, 14 days) could affect the peripheral nervous system of male SD rats, such as reducing velocity of caudal nerve conduction, contracting Schwann cell processes in unmyelinated fibers (Laham, S, Szabo, J and Long, G, 1983). Healy et al. found that TBP exposure could increase the salivary secretion of SD rats, prolong the stimulation response time, and reduce the activity ability and forelimb grip strength (Healy, C E, Beyrouty, P C and Broxup, B R, 1995). Hardos et al. found that TBP exposure could inhibit the activities of AChE and butyryl cholinesterase (BuChE) in aircraft maintenance workers, showing certain neurotoxicity (Hardos, J E, Rubenstein, M and Pfahler, S, et al., 2020). Carrington et al. reported that the sublethal dose of TBP (1500 mg/kg) could increase the activity of BuChE in hens’ plasma by 2 ~ 3 times, while the activities of AChE and neuropathy target esterase did not change, and TBP did not damage the function of nervous system (Carrington, C D, Lapadula, D M and Othman, M, et al., 1990). In conclusion, whether TnBP will cause neurotoxicity and the concrete mechanisms of neurotoxicity are not exactly same in different biological species. Therefore, to clarify the impacts of TnBP on environmental hygiene and human survival, more explorations on TnBP neurotoxicity are urgently needed.

C. elegans is a typical model organism in the field of life science due to its short life cycle, short life span, simple nervous system structure, clear genetic information, easily culture and other characteristics (Wang, D, 2016). The nervous system of nematodes contains 302 neurons. On the basis of the developmental cell lineage, the structure, location and connection between each neuron in C. elegans are very clear (Sulston, J E, 1983), so the damage mechanisms of the neurobehaviors can be explored through the damage of neurons. The neurotransmitters, vesicular circulation and synaptic transmission of nematodes have already been elucidated, which makes the exploration of nerve injury more favorable (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022). Nematodes contain diverse classical neurotransmitters, including dopamine, glutamic acid, GABA, acetylcholine, neuropeptide, 5-hydroxytryptamine, etc., so the damage and its mechanisms of nervous system can be studied according to neurotransmitters related molecules simultaneous express green fluorescence protein (GFP). The pollutant molecules can be quickly dispersed and fused into the nervous system after being absorbed, because nematodes lack blood-brain barrier. Therefore, C. elegans are especially applicable to explore the effects on neurobehavior. The stress-related mitogen activated protein kinase (MAPK) signal cascades
can be classified into three subfamilies: p38 MAPK signal, c-junn-terminal kinase (JNK) signal, and ERK signal (Li, W, Wang, D and Wang, D, 2018). In fact, the term “stress-activated MAPK” was a misnomer triggered by early studies where p38 MAPK signal and JNK signal were mainly activated by some harmful stimulating factors (osmotic stress, heat shock, cytokines, etc.) (Wagner, E F and Nebreda, A R, 2009). At present, people have realized that p38 MAPK signal pathway not only regulates stress-related signals, but also regulates normal signals (Wagner, E F and Nebreda, A R, 2009; Villanueva, A, Lozano, J and Morales, A, et al., 2001). As a central signal hub, MAPK signal pathway can transduce extracellular signals and trigger corresponding cellular responses (Andrusiak, M G and Jin, Y, 2016). In C. elegans, p38 MAPK signal is a sensor of extracellular stimuli, which allows nematodes to adapt to environmental toxins or pressures, such as engineered nanomaterials, pathogenic microorganisms and simulated microgravity (Li, W, Wang, D and Wang, D, 2018; Zhao, Y, Zhi, L and Wu, Q, et al., 2016). Pmk-1, sek-1, and nsy-1 are responsible for encoding a MAPK, a kinase of MAPK and a kinase of MAPK kinase, respectively, which constitute the core of p38 MAPK signal pathway (Andrusiak, M G and Jin, Y, 2016). The main purpose of the study was to explore the impacts of TnBP on neurobehavior of C. elegans and its mechanisms. The research results laid a foundation for the study of the risks of organic phosphate flame retardants to environmental hygiene and human survival.

2. Methods And Materials

2.1. Strains and culture

The lethality, growth and development toxicity, and motor behaviors toxicity of TnBP were evaluated by making use of wild-type C. elegans N2. The toxic impacts of TnBP on dopaminergic neurons, glutamatergic neurons and GABA neurons were assessed by making use of BZ555, DA1240 and EG1285 nematodes (Caenorhabditis Genetics Center, USA) which are the transgenic nematode strains, respectively. At the same time, whether the pmk-1 mutants (Caenorhabditis Genetics Center, USA) were susceptible to the harmful effects induced by TnBP was measured. For maintenance of C. elegans and synchronization of L1 stage larvae, please refer to relevant literature (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022).

2.2. Measurement of half lethal concentration (LC$_{50}$)

L1 larvae of wild-type N2 C. elegans (Caenorhabditis Genetics Center, USA) were exposed to TnBP of 100, 200, 300, 350 and 400 mg/L (CAS: 78-43-3, 99% pure, Toronto Research Chemicals, Canada) for 72 hours. The half lethal dose was used to determine the LC$_{50}$. The experiment was repeated 3 times.

2.3. Evaluation of growth and development status

L1 wild-type N2 C. elegans were exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours, and then approximately 20 nematodes were stochastically selected from each group to observe the changes of growth and development indicators (body length and body width) in nematodes. The experiment was repeated three times.
2.4. Evaluation of motor behaviors

The motor behaviors of nematodes include head swings, body bends, pump contraction times (feeding frequency), and so on. L1 wild-type N2 *C. elegans* were exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours and then approximately 20 nematodes were stochastically selected from each group to observe and record the changes of motor behaviors in *C. elegans*. Determination of head swings: transferred the nematodes to NGM medium excluding OP50 and let it recovery for 1 min, then observed and recorded the number of times of *C. elegans* head swings within 1 min with the help of the differential interference contrast microscope (Olympus SZ61, Japan). Determination of body bends: took the pharyngeal pump direction of *C. elegans* as the x-axis, and the body bends could be expressed as the changes of the body in the y-axis direction when crawling. After the exposure, the nematodes were transferred to NGM medium excluding OP50. After 1 min free crawling recovery, approximately 20 nematodes were stochastically selected from each group, and the number of times of body bends of the nematodes within 20 s was recorded. Pump contraction times (feeding frequency): can be interpreted as the regular shrinking of the pharyngeal pump in nematodes. Because nematodes ingest food from the outside through pumping contraction, the pump contraction times are also called feeding frequency. Please refer to the existing literature for the specific method of measuring the pump contraction times(Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022).

Chemical trend was a learning behavior that links water-soluble chemicals with hunger. Wild-type nematodes tend to NaCl under normal circumstances, while avoid NaCl under starvation circumstances. The determination steps of chemical trend index were as follows: firstly, took two points C and D (with a spacing of 4cm) on the test dish, cut two cylindrical agar blocks from the agarose medium (excluding net sodium chloride) with a circular plug and placed them at points C and D. The NGM medium block with a volume of 1cm$^3$ (containing 100 mM NaCl (Biosharp, China)) was placed at point C, but not at point D. The test dish was put in a 4 ℃ refrigerator passing the night to get a consecutive sodium chloride gradient, and the NGM medium was removed the next day. Secondly, *C. elegans* exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours were transferred to NGM medium containing sodium chloride but not OP50 to starve for four hours. Thirdly, took any point on the vertical line of the line segment linking point C with point D as the starting point of nematode movement, and about 50 starved nematodes were transferred to this point. A drop of levamisole (Aladdin, China) of 0.5 mol/L was dropped at point C and point D, respectively. Let *C. elegans* crawl in the 20 ℃ incubator for 30 minutes. Then, the numbers of nematodes in area C and D were recorded as NC and ND, respectively. Then the chemical trend index could be calculated according to chemical trend index $= \frac{(NC - ND)}{(NC + ND)}$. The experiment was repeated three times. The experimental distribution diagram for determining the chemical trend index was shown in Fig. 2.

2.5. Evaluation of effects on neurons

BZ555, DA1240 and EG1285 are transgenic nematodes whose dopaminergic neurons, glutamatergic neurons and GABA neurons were marked by GFP, respectively. Their structures can be observed through
fluorescence microscope. Therefore, the potential impacts of TnBP on dopaminergic neurons, glutamatergic neurons as well as GABA neurons were explored by making use of BZ555, DA1240 and EG1285 nematodes, respectively (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022). Each transgenic nematode strain was firstly exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours, and then was diverted to a centrifuge tube to recover for 10 min. Discarded the supernatant, washed the OP50 attached to the nematodes with M9 buffer, and then anesthetized the nematodes with levamisole. The anesthetized nematodes were diverted to a clean slide, then stochastically photographed approximately 20 nematodes in each group through a fluorescent microscope. The photos were processed by Image J software (National Institutes of Health, USA) to get mean fluorescence intensity. Attention should be paid to ensure the consistency of the shooting and processing parameters of all photos, when taking and processing photos. This experiment was repeated three times.

2.6. Evaluation of oxidative stress

N2 C. elegans were exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours, washed with M9 buffer and diverted to 1µM CM-H2DCFDA, kept away from light for 3 hours (Qu, M, Li, D and Qiu, Y, et al., 2020). Then the nematodes were picked onto the 2% agarose gel pad and were anesthetized with levamisole. Then approximately 20 nematodes were stochastically photographed from each group with fluorescence microscope. Under the fluorescence microscope, ROS showed a bright green mark. When taking pictures with a fluorescence microscope, attention should be paid to fast focusing, and forbid delaying excessively long, to avoid the influence of fluorescence quenching on experimental results. The experiment was repeated three times.

2.7. Expression levels of genes bound up with p38MAPK signal pathway and mitochondrial oxidative stress

Please refer to the existing literature for the determination method of related genes expression levels (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022).

2.8. Sensitivity examination of pmk-1 mutant to TnBP toxicity

Took the changes of motor behaviors as the endpoint of toxicity assessment, the regulatory effect of p38 MAPK signal pathway on TnBP toxicity for nematodes was measured. The pmk-1 mutant nematodes (KU25) were exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours and then approximately 20 C. elegans were stochastically picked from each group to record the changes of nematode head swings, pump contraction times, and body bends. The experiment was repeated three times.

2.9. Statistical analysis

Differences between groups were measured by one-way ANOVA provided by GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). All experimental data were expressed as mean ± standard
deviation. It was considered that the difference was statistically significant, while compared to the control group ($P \leq 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, when compared with the control.

3. Results

3.1 LC$_{50}$ of TnBP to nematodes

It could be seen from the Fig. 3 that the survival rate of nematodes reduced as the exposure concentrations of TnBP rises, with a LC$_{50}$ of 303.3 mg/L.

3.2. Effect of TnBP on the growth and development of nematodes

According to the LC$_{50}$, the exposure concentration of nematodes was finally determined as 0, 0.1, 1, 10, 20 mg/L, and the concentration in the covered environment was considered. It could be seen from the Fig. 4 that the body length and body width of *C. elegans* were significantly inhibited to varying degrees in each exposed group, while compared to the control group ($P < 0.05$).

3.3. Effect of TnBP on motor behaviors of nematodes

It could be seen from the Fig. 5 that the head swings of nematodes were significantly restrained ($P < 0.05$) in 1, 10 and 20 mg/L group; The body bends of nematodes were significantly inhibited ($P < 0.05$) in 10 and 20 mg/L group; The pump contraction times of nematodes were significantly inhibited ($P < 0.05$) in all exposed groups; From 1mg/L, the chemical trend index decreased significantly ($P < 0.05$), and the chemical trend index of 1, 10 and 20 mg/L group were all negative, among which the most significant decrease occurred in 20 mg/L group, compared with the control.

3.4. Damage of TnBP to nematode related neurons

*DAT-1* gene in nematodes is primarily in charge of encoding transporters of dopamine neurotransmitters, and *DAT-1::GFP* is able to be specially expressed in any dopaminergic neuron(Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J,2022). So, the transmission of dopamine in nematodes can be analyzed through the fluorescence intensity of transgenic nematode BZ555. The level of GFP and the form of GABA neuron were measured by the fluorescent intensity of the co-expression of GFP and *unc-47*. It could be seen from the Fig. 6 that the relatively fluorescent intensity was significantly enhanced to varying degrees in each exposed group, compared with the control ($P < 0.05$).

3.5. Effect of TnBP on oxidative stress level of nematodes

It could be seen from the Fig. 7 (b) that the ROS level of nematodes rose significantly from 1mg/L ($P < 0.05$). It could be found from the Fig. 7 (c) that the expression of the *mev-1* and *gas-1* significantly rose in
0.1, 1 and 10 mg/L group, while increased in the 20mg/L group, compared with the control ($P < 0.05$).

### 3.6. The expression of genes (pmk-1, sek-1 and nsy-1) related to p38 signal pathway

It could be seen from the Fig. 8 that the expression levels of *pmk-1, sek-1* and *nsy-1* significantly rose, compared to the control ($P < 0.05$).

### 3.7. Sensitivity of pmk-1 mutant C. elegans to TnBP exposure

Normally, there are no changes detected in motor behaviors in the *pmk-1* (KU25) mutant *C. elegans* (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022). After KU25 mutant nematodes and N2 nematodes were exposed to TnBP of 10mg/L for 72 hours, it could be seen from the Fig. 9 (a) that the head swings of KU25 and N2 *C. elegans* rose, compared to the control group ($P < 0.05$), and the rise of KU25 mutant *C. elegans* was more significant, compared to the N2 *C. elegans* ($P < 0.05$); It could be seen from the Fig. 9 (b) the body bends of KU25 and N2 nematodes had no obvious changes ($P > 0.05$); It could be seen from the Fig. 9 (c) the pump contraction times of KU25 and N2 *C. elegans* reduced significantly ($P < 0.05$).

### 4. Discussion

Although there were currently some studies on the harmful impacts of OPEs, such as the impacts of TDCPP on the neurobehavior of *C. elegans*, TnBP on the reproductive behavior of *C. elegans* and the neurobehavior of cultured PC12 cells. However, there was still a lack of research on the latent neurotoxicity of TnBP, and the research on the mechanisms of toxicity was even less. With the natural advantages of studying neurotoxicity, *C. elegans* were used to explore the neurotoxicity of TnBP in this study.

In this study, the growth and development of nematodes (body length and body width) were significantly inhibited by TnBP.

Our results showed that TnBP exposure could markedly increase the head swings and body bends, reduce the pump contraction times and chemical trend index in *C. elegans*. We found that there was a opposite variable trend between the head swings as well as body bends and pump contraction times as well as chemical trend index. It was reported that the feeding behavior was mainly regulated by serotonin, while head swings and body bends were controlled by additional neurons in *C. elegans* (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022). That was to say, there were different regulatory mechanisms among the pump contraction times, head swings and body bends, which could partly explain why the head swings and body bends increased, while the pump contraction times decreased. At the same time, it was speculated that the decrease of pump contraction times could lead to
the inhibition of nematode growth and development to a certain extent, because pump contraction reflects the feeding frequency of nematodes. Low feeding frequency might lead to growth retardation come very naturally. In addition, in this study, we also found that TnBP could significantly increase the number of dopaminergic neurons, glutamatergic neurons and GABA neurons in nematodes. As we all know, dopamine and glutamate are excitatory neurotransmitters. Therefore, we speculated that the activities of dopamine and glutamatergic neurons were one of the reasons why TnBP increased the head swings and body bends in *C. elegans*. The neurons in the nematodes can be divided into head sensory neurons, intermediate and motor neurons, of which the motor nerves can be divided into head motor nerves and body motor nerves. The nerve rings of the head mainly govern the muscle cells of the head, and the nerve rings of the ventral cord mainly govern the muscle cells of the body wall. The pharyngeal nervous system of nematodes is relatively independent, including 20 neurons. The correlation between pharyngeal nerves and head as well as somatic nerves of nematode is relatively small, which can explain why the variable trend of head swings and body bends of nematode was inconsistent with that of pump contraction times to a certain extent. *C. elegans* has 26 GABA neurons, which mainly function at the neuromuscular junction. GABA in *C. elegans* has the functions of both excitatory neurotransmitters and inhibitory neurotransmitters, among which the inhibitory GABA motor neurons regulate the body bends of *C. elegans*. When the muscles in one side of *C. elegans* body contract and the abdominal wall and dorsal wall muscles of the other side relax under the control of inhibitory GABA nerve, the sinusoidal movement of the body from head to tail, namely body bends, can be completed. It was speculated that GABA might have a positive regulatory effect on body bends. Therefore, it could be inferred that the increase of GABA neurons in this experiment was one of the reasons for the increase of the body bends of nematodes. In *C. elegans*, about 500 ~ 1000 G protein coupled receptors were expressed in chemosensory neurons, which became possible chemosensory receptors for external environmental stimuli or toxic and harmful substances, directly affecting chemical tropism. However, the mechanisms of TnBP causing the decrease of nematode chemical trend index still need further study.

ROS refers to oxygenated compounds (such as peroxides, superoxide anions, hydroxyl radicals, etc.) produced during aerobic metabolism. Oxidative stress will occur when the production of ROS exceeds the ability of organisms against oxidation, which is a latent toxic mechanism by which environmental risk factors can directly or indirectly cause toxicity (Mates, J M, Segura, J A and Alonso, F J, et al., 2008), such as destroying the cell structure and causing the corresponding dysfunction of cells. For example, oxidative stress could damage lipids, protein, and DNA (Valko, M, Leibfritz, D and Moncol, J, et al., 2007; Yan, S, Wang, Q and Yang, L, et al., 2020). DNA damage could lead to cell cycle arrest, promote the release of apoptotic factors, thus causing cell apoptosis, and eventually causing body death (Yan, S, Wang, Q, Yang, L and Zha, J, 2020). In nematodes, the *mev-1* and *gas-1* are very important for against oxidative stress (Wu, Q, Zhou, X and Han, X, et al., 2016). The *mev-1* and *gas-1* encode the integral membrane protein of mitochondrial respiratory chain complex II and mitochondrial complex I, respectively, both of which play an indispensable role in oxidative phosphorylation (Yin, J, Liu, R and Jian, Z, et al., 2018), and these two genes are related to the production of ROS in mitochondria of *C. elegans*. Zhang et al. found that exposure to TnBP of 0.1µg/L ~ 1000µg/L for 72 hours could significantly increase the ROS and
reduce the expression of *mev-1* and *gas-1* in *C. elegans* (Zhang, H, Liu, T, Song, X, Zhou, Q, Tang, J, Sun, Q, Pu, Y, Yin, L and Zhang, J, 2021). Kayser et al. found that mitochondrial function decreased, ROS increased in nematode mutants of *mev-1* and *gas-1* (Kayser, E B, Morgan, P G and Hoppel, C L, et al., 2001). Liu et al. also found that TnBP could induce the production of excessive ROS, thereby causing oxidative injury to *Phaeodactylum tricornutum* (Liu, Q, Tang, X and Wang, Y, et al., 2019). In this experiment, we also found that exposure to TnBP could induce overproduction of ROS in nematodes, significantly inhibit the expression of *mev-1* and *gas-1* at low concentrations (0.1, 1 and 10mg/L) \((P<0.05)\), while significantly increase their expression at high concentrations (20mg/L) \((P<0.05)\). Therefore, we speculated that the possible mechanism by which TnBP increased the production of ROS in *C. elegans* at low concentrations was to inhibit the expression of *gas-1* and *mev-1*. However, although TnBP at high concentration increased the expression of *mev-1* and *gas-1*, the production of ROS in nematodes still significantly rose, when compared to the control \((P<0.05)\). Therefore, it could be speculated that the mechanisms by which TnBP rose ROS level in *C. elegans* were not limited to affecting the expression of *mev-1* and *gas-1*, but there might also be other mechanisms, such as affecting the expression of other oxidative stress related genes. 

Large amount of oxygen consumption and high-speed metabolic activity make the brain more vulnerable to neurotoxicity induced by ROS (Mao, Z, Zheng, Y L and Zhang, Y Q, 2010). Therefore, it was speculated that TnBP might produce neurotoxicity by increasing the oxidative stress level of nematodes. Yang et al. found that TCEP and trimethyl phosphate produced neurotoxicity to earthworms mainly through oxidative stress (Yang, Y, Xiao, Y and Chang, Y, et al., 2018). At the same time, some studies showed that ROS levels were closely related to neurotoxicity (Abramov, A Y, Scorziello, A and Duchen, M R, 2007). In conclusion, TnBP might directly produce neurotoxicity by increasing the level of oxidative stress in nematodes or indirectly produce neurotoxicity by inducing apoptosis.

As mentioned earlier, in *C. elegans*, p38 MAPK signal, as a sensor for extracellular stimuli, allows cells to adapt to environmental toxins or pressures, such as engineered nanomaterials, pathogenic microorganisms, and simulated microgravity. Sanchez et al. found that the neurotoxicity of CXCL12 could be abrogated through direct block p38 MAPK signal pathway (Sanchez, A B, Medders, K E and Maung, R, et al., 2016). Tang et al. found that TDCPP could produce neurotoxicity by activating p38 MAPK signal pathway in *C. elegans* (Tang, J, Li, J, Zhou, Q, Kuerban, G, Qin, J, Zhang, H, Sun, R, Yin, L, Pu, Y and Zhang, J, 2022). Lim et al. found p38 MAPK signal pathway could save the decrease in reproductive capacity of *C. elegans* induced by silver nanoparticles (Lim, D, Roh, J Y and Eom, H J, et al., 2012). Li et al. observed that the p38 MAPK signal pathway might mediate a protective mechanism, thereby increasing the resistance of *C. elegans* to harmful effects (such as excessive production of intestinal ROS, shortening of life span, etc.) induced by simulated microgravity (Li, W, Wang, D and Wang, D, 2018). These studies showed that the sensitization of p38 MAPK signal pathway might be a toxic mechanism or a protection mechanism for various signals, including the normal as well as adverse signals. In this study, we found that TnBP significantly rose the expression of genes (*pmk-1*, *nsy-1* and *sek-1*) related to p38 signal pathway. In our validation test, we found that the susceptibility of *pmk-1* mutants to the neurotoxicity induced by TnBP was higher than that of wild-type N2 *C. elegans*, when used motor behaviors (head swings, body bends, pump contraction times) as a endpoint of adverse effects.
evaluation. Therefore, it was suggested that TnBP had neurotoxicity to *C. elegans* and the sensitization of p38 MAPK signal pathway might mediate a protective mechanism for *C. elegans* against the harmful effects induced by TnBP exposure. The results provide a vital basis for revealing the impacts of organic phosphate flame retardants on environmental hygiene and human survival.

5. Conclusion

In summary, we here explored the lethality, growth and development toxicity, neurotoxicity, and its mechanisms (such as active oxidative stress, the expression of related genes, etc.) of TnBP exposure for *C. elegans*. We found that the growth and development were significantly inhibited, the motor behaviors were affected (the head swings and body bends increased, the pump contraction times and chemical trend index reduced), the production of related neurotransmitters (dopamine, glutamate and GABA) and the production of ROS were risen, the expression levels of mitochondrial oxidative stress related genes were altered, the expression levels of genes related to p38 MAPK signal pathway were risen, under the exposure of TnBP of higher environmental related concentrations, in *C. elegans*. Meanwhile, the pmk-1 mutants led to the sensibility to neurotoxicity induced by TnBP exposure. Therefore, it was suggested that TnBP had neurotoxicity to *C. elegans*, the oxidative stress might be one of the mechanisms of neurotoxicity induced by TnBP. In addition, the sensitization of p38 MAPK signal pathway might be a protection mechanism for *C. elegans* against the harmful effects induced by TnBP exposure. The results provide vital information for clarifying the impacts of organic phosphate flame retardants on environmental hygiene and human survival.

**Declarations**

**Ethical Approval**

This declaration is “not applicable”.

**Competing interests**

The authors have no relevant financial or non-financial interests to disclose.

**Authors’ contributions**

Jielin Tang and Jinyan Qin were responsible for experimental operation, data analysis and writing article; Guzailinuer Kuerban, Jiayi Li and Qinyu Zhou were responsible for determining the topic selection and experimental operation; Hongdan Zhang and Rongli Sun participated in experimental operation; Lihong Yin and Yuepu Pu were responsible for revising article; Juan Zhang were responsible for nal revision.

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Availability of data and materials

The datasets generated or analyzed and the materials used during this study are available from the corresponding author on reasonable request.

References


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The evaluation of the motor behaviors: the head swings (a), body bends (b), pump contraction times (c) and chemical trend index (d) of nematodes exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours.
Figure 6

The evaluation of the neurons damage of *C. elegans* exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours: the fluorescence photos of dopaminergic neurons (a), glutamatergic neurons (b) and GABA neurons (c), the relatively fluorescent intensity of dopaminergic neurons (d), glutamatergic neurons (e) and GABA neurons (f)
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The levels of ROS and the expression of relevant genes: the fluorescent photos (a), relatively fluorescent intensity of oxidative stress (b) and the expression levels (c) of genes (*mev-1* and *gas-1*) related to mitochondrial oxidative stress of nematodes exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours.
Figure 8

The expression of p38 signal pathway related genes (*pmk-1, sek-1 and nsy-1*) of *C. elegans* exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours
Figure 9

The comparison of sensitivity of wild-type N2 and KU25 *C. elegans* to TnBP toxicity: the head swings (a), body bends (b) and pump contraction times (c) of N2 and KU25 nematodes exposed to TnBP of 0, 0.1, 1, 10 and 20 mg/L for 72 hours.