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Reproductive toxicity and underlying mechanisms of di(2-ethylhexyl) phthalate in nematode *Caenorhabditis elegans*

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ABSTRACT

DEHP (di(2-ethylhexyl) phthalate) is an endocrine disruptor commonly found in plastic products that has been associated with reproduction alterations, but the effect of DEHP on toxicity is still widely unknown. Using DEHP concentrations of 10, 1, and 0.1 mg/L, we showed that DEHP reduced the reproductive capacity of *Caenorhabditis elegans* after 72 hr. of exposure. DEHP exposure reduced the reproductive capacity in terms of decreased brood sizes, egg hatchability (0.1, 1 and 10 mg/L), and egg-laying rate (1 and 10 mg/L), and increased numbers of fertilized eggs in the uterus (1 and 10 mg/L). DEHP also caused damage to gonad development. DEHP decreased the total number of germline cells, and decreased the relative area of the gonad arm of all exposure groups, with worms in the 1 mg/L DEHP exposure group having the minimum gonad arm area. Additionally, DEHP caused a significant concentration-dependent increase in the expression of *unc-86*. Autophagy and ROS contributed to the enhancement of DEHP toxicity in reducing reproductive capacity, and glutathione peroxidase and superoxide dismutase were activated as the antioxidant defense in this study. Hence, we found that DEHP has a dual effect on nematodes. Higher concentration (10 mg/L) DEHP can inhibit the expression of autophagy genes (*atg-18*, *atg-7*, *bec-1*, *lgg-1* and *unc-51*), and lower concentrations (0.1 and 1 mg/L) can promote the expression of autophagy genes. Our data highlight the potential environmental risk of DEHP in inducing reproductive toxicity toward the gonad development and reproductive capacity of environmental organisms.

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Introduction

Di(2-ethylhexyl) phthalate (DEHP), as one of the most frequently detectable phthalate esters (PAEs), is broadly applied as a plasticizer in polyvinyl chloride (PVC) production (Wu et al., 2018). Concurrently, we are continuously exposed to DEHP-containing plastic products, such as plastic bottles, toys, packaging materials, and even medical and clinical devices (Hu et al., 2016; Latini et al., 2010). Due to its physical bond with the polymeric matrix, PAEs can be easily released into the environment in the process of manufacture, use, and disposal (Net et al., 2015). So far, a growing number of studies have indicated that DEHP can be detected in various environmental matrices, such as indoor and outdoor air, water bodies, soil sediments, and even biota (Ding et al., 2019; Fossi et al., 2012; Huang et al., 2008; Kashyap and Agarwal, 2018; Raffy et al., 2017; Selvaraj et al., 2015; Zhan et al., 2016; Zhang et al., 2018c). In addition, DEHP and its metabolites have also been detected in human urine, breast milk, blood and saliva (Hines et al., 2009; Silva et al., 2005). Hence, a series of in-depth studies should be conducted to uncover the potential risks caused by DEHP.

DEHP exposure may induce various toxic effects in animals and humans, such as reproductive toxicity, neurotoxicity and carcinogenesis (Martinez-Arguelles et al., 2013; Qiu et al., 2020; Zhang et al., 2016). In addition, reproductive toxicity has been frequently reported after adult exposure to environmental and occupational doses of DEHP (Parra-Forero et al., 2019; Richardson et al., 2018). Meanwhile, a recent study using mice showed that exposure to DEHP could also alter the meiotic progression of fetal oocytes, oocytes apoptotic and expression of the related genes (Liu et al., 2017). Furthermore, exposure of pregnant women to DEHP at high concentrations may lead to low birth weight (Tsai et al., 2018). However, even though we know DEHP can induce reproductive toxicity, its underlying mechanisms still remain unclear.

In *Caenorhabditis elegans*, the best-characterized example of serotonin signaling in a genetically tractable model organism occurs in the egg-laying system (Schafer, 2005). Egg-laying results from the contraction of egg-laying muscles, which are stimulated by serotonin released from Hermaphrodite Specific Neurons (HSNs). *Unc-86* expression in the HSN motor neurons is a prerequisite for serotonin production by these cells (Desai et al., 1988; Sze et al., 2002). It is interesting to note that either increased expression levels in UNC-86/VP16 animals or decreased expression levels in *unc-86*-null mutants causes incorrect interpretation of guidance cues and results in neurite outgrowth defects (Sze et al., 2002). Research also shows that loss of wild-type UNC-86 or the presence of UNC-86/VP16 would result in decreased serotonin levels, since UNC-86 may normally repress the activity of genes that downregulate serotonin synthesis or upregulate serotonin release, with the consequence of decreasing serotonin levels in the HSN (Sze et al., 1997). In addition to positive autoregulation, *unc-86* also negatively autoregulates its expression (Baumeister et al., 1996). A study showed that wild-type animals carrying *unc-86*/VP16 are egg-laying defective and do not accumulate serotonin, suggesting that increased expression levels in UNC-86/VP16 cause serotonergic defects in the HSN neurons (Sze et al., 1997).

C. elegans, simple but helpful, has been extensively used in genetic, molecular and toxicological studies due to its transparent body, short life cycle, fixed number of germ cells and distinct genetic background (Ferreira and Allard, 2015; Qu et al., 2019c; Qu and Wang, 2020). In addition, *C. elegans* has been applied as a classical model to detect reproductive toxicity and explore possible mechanisms (Du et al., 2015; Qu et al., 2019b; Yin et al., 2018; Yu et al., 2017). *C. elegans* is therefore a rational choice to test reproductive toxicity and explicate the underlying mechanisms of DEHP.

In the present study, we employed *C. elegans* as the model organism to analyze potential reproductive toxicity using multiple endpoints, such as brood size, egg-laying rate, amount of germ cells, and so forth. Additionally, we also detected HSN function damage, autophagy, ROS level and expression of involved antioxidant defense system activities corresponding to reproductive toxicity. The data suggested that the damage caused by DEHP to reproductive function, HSN signaling, increased autophagy and ROS level will be helpful in understanding the possible reproductive toxicity of DEHP.

1. Materials and methods

1.1. Animal maintenance and exposure

Wild-type N2 and transgenic strains of *DA2123, adIs2122* [*lgg-1p*::GFP::*lgg-1*+*rol-6(su1006)*] and *CX5974, kyIs262[unc-86*::myr GFP+*odr-1*::RFP] were used in the present study. All these strains were purchased from the Caenorhabditis Genetics Center (CGC, University of Minnesota, Minneapolis, MN, USA), cultured and manipulated as previously described (Brenner, 1974). The afore mentioned nematodes were cultured on nematode growth medium (NGM, 2.5 g/L peptone, 3 g/L NaCl, 17 g/L Agar, 1 mL/L cholesterol (5 mg/L), 25 mL/L KPO_4 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, H_2O to 1 L), 1 mL/L $MgSO_4$ (120 g/L), 1 mL/L $CaCl_2$ (110 g/L))at 20°C with *Escherichia coli* OP50 as food source.

DEHP was obtained from J&K SCIENTIFIC LTD (Shanghai, China). DEHP was dissolved in DMSO (dimethyl sulfoxide, Sigma–Aldrich, USA, final concentration < 0.1%) to form the stock solution, and then was diluted in K-medium (2.4 g KCl, 3 g NaCl, H_2O to 1 L) to prepare working solutions with concentrations of 0.1, 1.0 and 10.0 mg/L. The hermaphrodite worms were lysed by a mixed hypochlorite solution (5% NaOH (200 g/L), 12% NaClO) to gain fertilized eggs and leave these eggs to incubate on OP50-free NGM medium at 20°C overnight. Age-synchronous populations of L1-larvae were transferred to NGM medium with OP50 to allow them to develop to L4, and then were collected for exposure. Age-synchronized L4 worms were transferred to worm liquid culture systems containing 200 μL 0, 0.1, 1.0, or 10 mg/L DEHP with sufficient food at 20°C.

1.2. Reproductive capacity assay

Brood size, egg-laying rate and amount of eggs in the uterus are viewed as classical endpoints to reveal the reproductive

capacity of worms (Qu et al., 2019b, 2019c). Brood size was considered as the amount of offspring after 72 hr. exposure of worms in this study. After exposure, at least 20 worms from each exposure group were transferred to new NGM plates in the presence of OP50 to count brood size, and the NGM plates with OP50 were refreshed daily. At least sixty nematodes were examined per treatment. Hatchability represents the ratio of progeny number to brood size. At least twenty nematodes were examined per treatment. Egg-laying rate was defined as the average total number of eggs (10 worms) per hour from the spawning period. After exposure, 10 animals from each exposure group were transferred to NGM agar plates and allowed to lay eggs for 4 hr. Thirty nematodes were examined per treatment. The amount of eggs in the uterus was regarded as the number of eggs in utero. A differential interference contrast (DIC) microscope was used to capture and count the eggs in the uterus. An Olympus SZ61 Stereo Microscope (Olympus, Japan) was used. Detection was repeated at least three times, with at least thirty nematodes examined per treatment.

1.3. Gonadal development assay

The total number of germline cells and the gonadal area were used to reflect gonadal development as in previous works (Qu et al., 2019c; Quevedo et al., 2007). 4,6-diamino-2-phenyl indole (DAPI) is a chemical dye that can strongly combine with DNA (Lant and Derry, 2014). After 72-hr exposures, at least 30 worms from each exposure group were washed three times using 90% alcohol and then incubated in DAPI (2 μg/mL) for 30 min without light, after which the gonadal development could be visualized by fluorescence microscopy. A ZEISS AXIO Imager.M2 (Carl Zeiss Jena, Germany) was used. Detection was repeated at least three times, and at least ninety nematodes were examined per treatment.

1.4. Detection of positive autophagy puncta

The structure of autophagic precursors and autophagic bodies can be observed and counted by observing the presence of GFP positive puncta labeled with autophagic vesicles (Melendez et al., 2003). In nematodes, transgenic strain *adIs2122* carrying LGG-1::GFP fusing protein are localized in lateral epidermal seam cells (Mosbech et al., 2013). After 24-hr exposure, *adIs2122* worms were collected and rinsed three times using M9 buffer, and at least 20 worms were picked randomly for the detection assay with the aid of a fluorescence microscope; a ZEISS AXIO Imager.M2 (Carl Zeiss Jena, Germany) was used. Detection was repeated at least three times, and at least sixty nematodes were examined per treatment.

1.5. Visualization of hermaphrodite specific neuron (HSN) deficiency

A transgenic strain of *kyIs262* nematodes was used to detect HSN damage in this study, as *unc-86*::*myr* GFP+*odr-1*::RFP could visualize both immature and mature HSNs (Shinkai et al., 2018). The GFP reporter enabled us to observe HSN cell bodies, which are normally located slightly posterior to the vulva (Moresco and Koelle, 2004). They extend axons that innervate the egg-laying muscles and express the neurotransmitter serotonin (Desai et al., 1988; Finney et al., 1988). *Unc-86* expression by the HSN motor neurons is a prerequisite for serotonin production by these cells (Desai et al., 1988; Sze et al., 2002). After 72-hr of exposure, *kyIs262* animals were collected and washed three times using M9 buffer, and at least 20 worms were picked randomly for detection per treatment. A ZEISS AXIO Imager.M2 (Carl Zeiss Jena, Germany) was used. Detection was repeated at least three times, with at least sixty nematodes examined per treatment.

1.6. Reactive oxygen species (ROS) detection

The fluorescent probe 2′,7′-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) was used to detect the intracellular ROS level in this study, as previously described (Qu et al., 2019a). After 72 hr of exposure to DEHP, worms were washed three times, and then 0.5 μL H_2DCF-DA was added into each Eppendorf tube to obtain a final concentration of 50 μmol/L. After a 2-hr incubation without light, animals were washed using M9 at least three times before ROS detection with the aid of a fluorescence microscope. At least 20 worms were measured for each treatment. A ZEISS AXIO Imager.M2 (Carl Zeiss Jena, Germany) was used. Detection was repeated at least three times, and sixty nematodes were examined per treatment.

1.7. Measurement of superoxide dismutase (SOD) and glutathione peroxidase (GPX)

After 72 hr of exposure to DEHP, nematodes were washed with M9 buffer, then the nematodes were homogenized for measurement of SOD and GPX. The homology kit of Beyotime Biotechnology (Nanjing, China) was used for SOD and GPX measurement according to the manufacturer's instruction. At least three independent replications were performed for each assay.

1.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

After exposure, nematodes were washed with M9 buffer at least 3 times in enzyme-free centrifugal tubes. The total RNA of nematodes in each group was isolated after treatment using Trizol (Sigma-Aldrich, St. Louis, MO, USA). 500 μL Trizol (Sigma Aldrich, St. Louis, MO, USA) was added to each Eppendorf tube respectively after discarding the supernatant. The RNA purity and concentration were controlled by the ratio of OD260/280. A Mastercycler gradient PCR system (Eppendorf, USA) was employed to synthesize cDNA with the reverse transcriptase reaction. The expression levels of involved genes were determined by a StepOnePlus™ real-time PCR system (Applied Biosystems, Carlsbad, USA) using the SYBR Green qRT-PCR master mix (Yeasen, Shanghai, China). *18s* was chosen as the reference gene and at least three replicates were performed in all reactions. Table 1 shows all the detailed sequences.

1.9. Statistical analysis

All results were plotted as mean ± standard error (SE). One-way analysis of variance (one-way ANOVA) and *Dunnett's t*-

Table 1 – Gene sequences.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
18s	TGGAGCTTGCGGCTTAAT	TTACCGATACCTTTCGGCATAG
unc-51	CGCCGGTGGTTCAGCGGATT	TATCCTGGGTGTCGGCGGGG
bec-1	ACGAGCTTCATTCGCTGGAA	TTCGTGATGTTGTACGCCGA
atg-18	CAGGAGCCGCAAGGAGTAAT	CGATTGGTTGCTTGCTTCGG
atg-7	CCAAAAGCTGTGGGATGGGA	GCGTTCCAGCACCAAGAATG
lgg-1	GCCGAAGGAGACAAGATCCG	GGTCCTGGTAGAGTTGTCCC

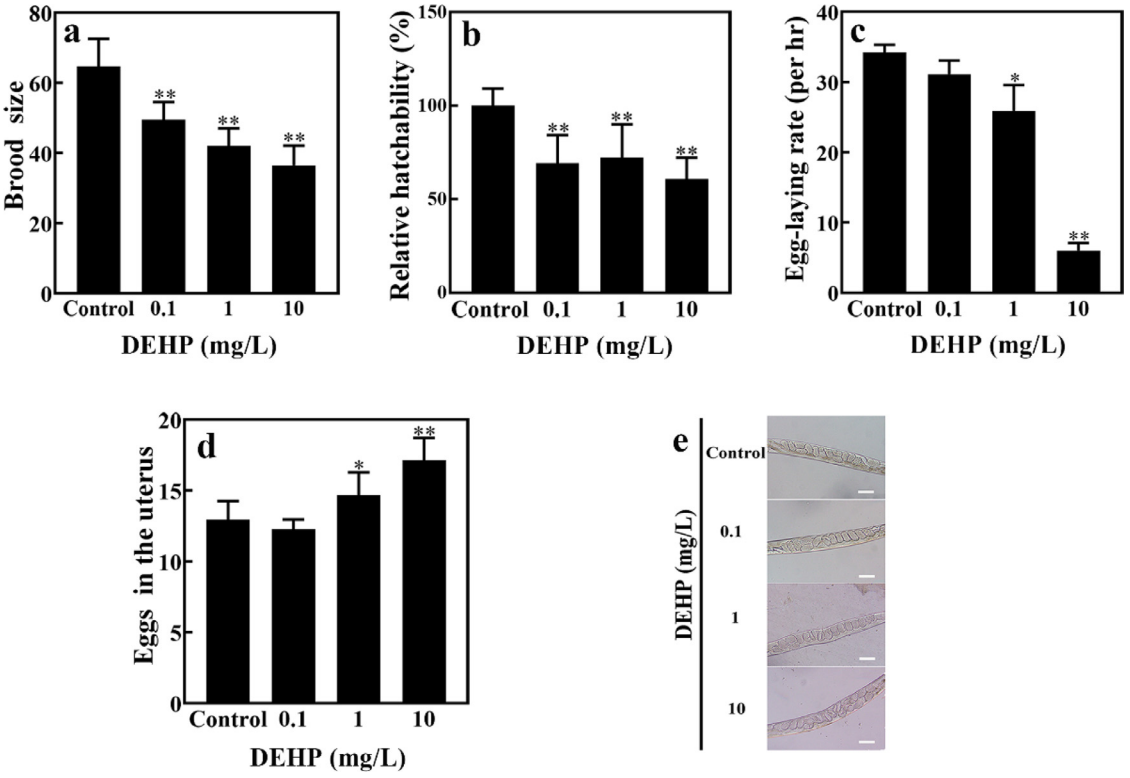

Fig. 1 – DEHP exposure-induced reproductive capacity deficiency in wild-type *C. elegans* after 72 hr. exposure. (a) reduction in brood size of wild-type *C. elegans*; (b) reduced hatchability in wild-type *C. elegans*; (c) decrease in egg-laying rate in wild-type *C. elegans*; (d) blocking of fertilized eggs in the uterus; (e) Fertilized eggs in the uterus. Bars represent means ± SD. * $P < 0.05$ vs control and ** $P < 0.01$ vs. control (if not specially indicated). Scale bars: 50 μm.

test was used to determine differences between both exposure groups and the control. Statistical analysis was accomplished using SPSS 20.0, and $P<0.05$ was considered statistically significant.

2. Results

2.1. *DEHP exposure induced reproductive capacity deficiency in wild-type* C. elegans

First, we investigated the effects of DEHP on reproductive toxicity in wild-type worms by employing the endpoints of brood size, egg-laying rate and amount of fertilized eggs in the uterus. After exposure, DEHP (≥ 0.1 mg/L) could significantly reduce brood size and egg hatchability in wild-type worms (Fig. 1a and b, $P<0.05$). Different from this, a decline in egg-laying rate could be only observed in the 1 and 10 mg/L DEHP exposure groups (Fig. 1c, $P<0.05$). Similarly, DEHP (>0.1 mg/L) could meaningfully block fertilized eggs in the uterus (Fig. 1d and e, $P<0.05$). Moreover, worm-bags were observed in the 1 and 10 mg/L DEHP exposure groups. Our data showed that DEHP exposure induced reproductive capacity deficiency in wild-type *C. elegans*.

2.2. *DEHP exposure affected gonadal development in wild-type* C. elegans

Next, we further visualized the total germline cells and gonadal area (identified by the red line area) using DAPI stain. After the exposure, DEHP significantly reduced the amount of total germline cells per gonad, and the 10 mg/L DEHP exposure

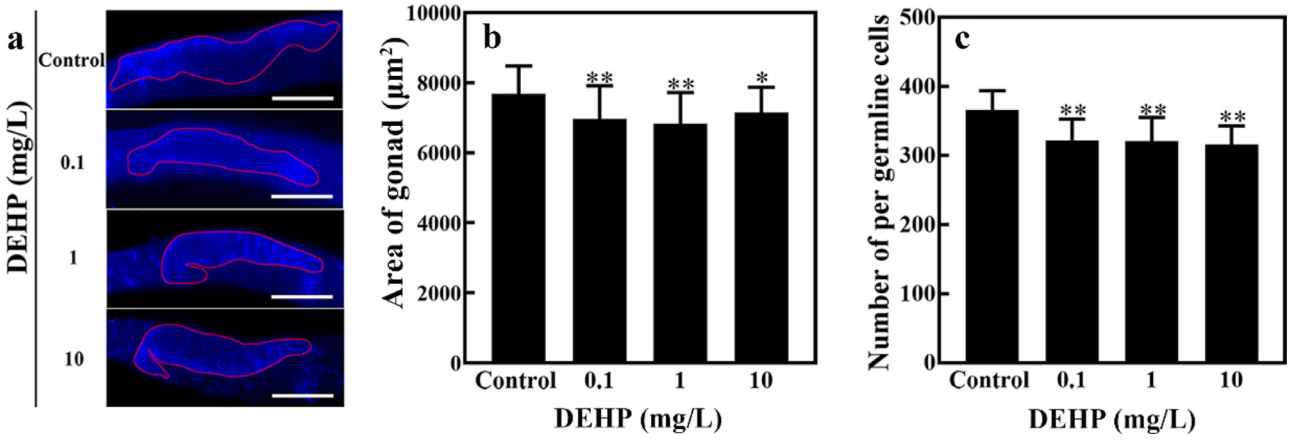

Fig. 2 – DEHP exposure effects on gonadal development in wild-type *C. elegans*. (a) represents the DAPI result from the control group, 0.1, 1 and 10 mg/L DEHP exposure groups, respectively. (b) Comparison of area of gonad arms for different groups. (c) Comparison of the number of total germline cells per gonad in different groups. Bars represent means ± SD. * P <0.05 vs. control and ** P <0.01 vs. control (if not specially indicated). Scale bars: 100 μm.

group had the minimum number of germline cells per gonad (Fig. 2c, $P<0.01$). Differently, DEHP significantly decreased the relative area of the gonad arm, with the greatest decrease observed in the 1 mg/L DEHP exposure group (Fig. 2b, $P<0.05$).

2.3. *DEHP exposure dynamically inhibited HSN function*

To further understand the underlying mechanism for the reduction in egg laying, we next detected HSN, which innervates the muscle involved in egg-laying, stimulating contraction (Bany et al., 2003; Moresco and Koelle, 2004). *Punc-86*::myr gfp marks HSNs in *C. elegans* and the expression of GFP fusion protein is reflected in the HSN as green fluorescence (Shinkai et al., 2018). Increased expression levels in *unc-86*/VP16 caused serotonin levels to decrease in the HSN (Sze et al., 1997). After DEHP exposure, the fluorescence intensity of the HSN was significantly increased, suggesting that DEHP may increase the expression of *unc-86* to cause HSN defects in serotoninergic signaling (Fig. 3a and b, $P<0.01$).

2.4. *DEHP exposure dynamically affected autophagy*

Then, we explored the autophagy level of nematodes after DEHP exposure. DEHP induced obvious autophagy in the 0.1 and 1 mg/L DEHP exposure groups (Fig. 4a). By contrast, the 10 mg/L exposure group showed no statistically significant differences compared to control (Fig. 4a). The data showed that lower concentrations of DEHP promoted autophagy while higher concentrations of DEHP restrained autophagy in *C. elegans*.

Autophagy is essential for survival, differentiation, development and homeostasis (Alberti et al., 2010). It is also involved in determining cell size (Aladzsity et al., 2007). LGG-1::GFP was used as a marker to reflect the induction of autophagy in *C. elegans* (Min et al., 2019). LGG-1, an ortholog of ATG8/LC3, associates with autophagosomal membranes upon induction of autophagy (Zhang et al., 2015). DEHP exposure could measurably induce the autophagy genes *atg-18, atg-7* and *unc-51*, which significantly increased in the 0.1 and 1 mg/L

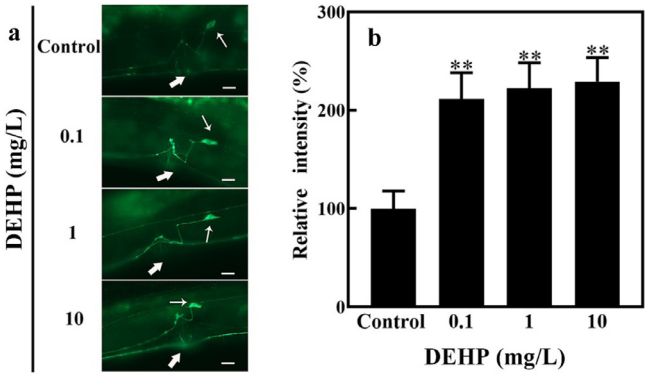

Fig. 3 – DEHP exposure-increased *unc-86* expression. (a) Represents the images of protein from control group and 0.1, 1 and 10 mg/L DEHP exposure groups, respectively. (b) Comparison of fluorescence intensity of HSN (Hermaphrodite Specific Neurons) in different groups. Exposure to DEHP was performed from L4 for 72 hr. Arrowheads indicate the vulva and thick arrows indicate the HSN. Bars represent means ± SD. ** P <0.01 vs. control (if not specially indicated). Scale bars: 20 μm.

exposure groups, and showed no significant difference in the 10 mg/L exposure group compared to control (Fig. 4b). However, *bec-1* and *lgg-1* significantly increased in the 0.1, 1 and 10 mg/L exposure groups compared the control group (Fig. 4b).

2.5. *Effect of DEHP exposure on ROS, SOD and GPX*

ROS is usually an important contributor to the reduction in reproductive capacity of *C. elegans* exposed to environmental toxicity. The fluorescent probe H_2DCF-DA was used to label the ROS level of worms exposed to DEHP (Fig. 5a). DEHP exposure significantly enhanced the ROS level in *C. elegans* (Fig. 5a and b). SOD significantly increased in the 0.1 and 10 mg/L exposure groups, but no significant increase was observed in

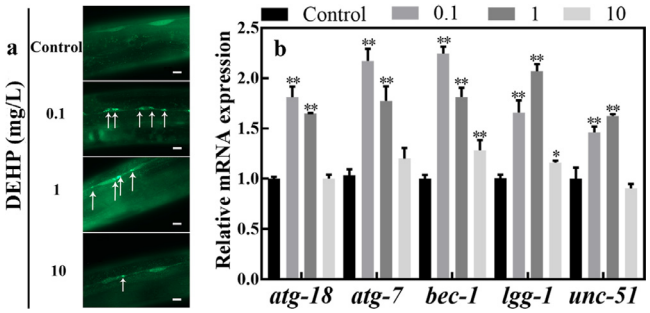

Fig. 4 – Dynamic effects of DEHP exposure on autophagy. (a) represents the autophagy results from the control group and 0.1, 1 and 10 mg/L DEHP exposure groups respectively; (b) Comparison of the expression of genes required for the control of autophagy. Exposure to DEHP was performed from L4-larvae to 24 hr. Thick arrows indicate GFP:: LGG-1 in the seam cell. Bars represent means ± SD. * P <0.05 vs. control and ** P <0.01 vs. control (if not specially indicated). Scale bars: 20 μm.

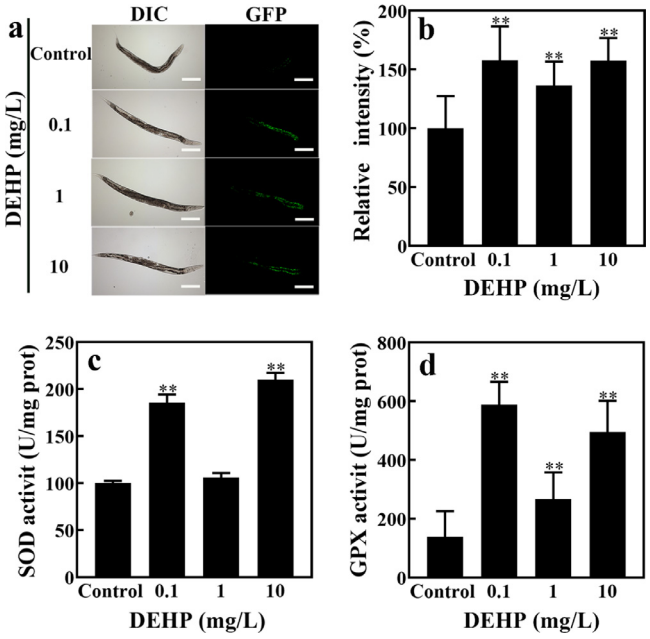

Fig. 5 – DEHP-enhanced reproductive toxicity by induction of ROS. (a) represents ROS result from control group and 0.1, 1 and 10 mg/L DEHP exposure groups, respectively. (b) Comparison of fluorescence intensity of ROS in different groups. (c) and (d) represent the SOD and GPX activities, respectively. Bars represent means ± SD. * P <0.05 vs. control and ** P <0.01 vs. control (if not specially indicated). Scale bars: 100 μm.

the 1 mg/L exposure group (Fig. 5c). The GPX activities significantly increased in the 0.1, 1 and 10 mg/L DEHP exposure groups (Fig. 5d). Hence, the increased SOD and GPX activities suggested that DEHP significantly activated the antioxidant defense systems.

3. Discussion

In *C. elegans*, a previous study demonstrated that exposure to DEHP could reduce the brood size and total germline cells (Yin et al., 2018), suggesting the potential toxicity of DEHP toward reproductive capacity. The decreased brood size, egg hatchability and egg-laying rate, increased number of fertilized eggs in the uterus and observation of the bag-of-worms phenotype in DEHP exposure further confirmed its growth development and reproductive toxicity in *C. elegans* (Fig. 1). In addition, we observed that exposure to DEHP also caused gonad development deficiency, reducing the total germline cells and germline area (Fig. 2b and c). Furthermore, the function of HSN, which controls the muscle cells whose contraction expels eggs (Collins et al., 2016; Desai et al., 1988), was significantly disordered by DEHP (Fig. 3a and b). The BOW increase in the worms implicates age-related degeneration of the egg-laying system, which may involve degeneration in the function of vulval muscles controlled by the HSN (Pickett and Kornfeld, 2013). Therefore, DEHP exposure distinctly induces both gonad development and reproductive toxicity.

In our study, DEHP at 1 mg/L could contribute to significant decreases in the egg-laying rate and increases in the numbers of eggs in the uterus (Fig. 1c and d), and markedly increased the occurrence of worm-bags in the 1 and 10 mg/L exposure groups. These results further suggest that 72-hr exposure to DEHP can lead to egg-laying defects. Meanwhile, we also performed the HSN assay, and the results indicated that DEHP brings about a meaningful increase in the fluorescence intensity of the HSN, suggesting that DEHP may significantly impact the function of the HSN by increasing the level of *unc-86* (Fig. 3a and b). Activation of egg laying depends on the HSNs, which release serotonin to initiate egg-laying behavior in *C. elegans* (Bany et al., 2003; Brewer et al., 2019), and *unc-86* is required for serotonin synthesis in the HSNs (Desai et al., 1988; Sze et al., 2002). The HSN neurons play a critical role in driving egg-laying behavior through immediate excitation of the vulva muscles and VC motoneurons (Zhang et al., 2008); therefore, the increased level of *unc-86* in the HSN will downregulate HSN serotonin levels as well as suppressing the egg-laying behavior of worms. Our data showed that DEHP possibly acted by increasing the expression of *unc-86* to decrease the HSN serotonin levels, with the consequences of increased fertilized eggs in the uterus and occurrence of the bag-of-worms phenotype to increase reproductive toxicity.

Previously studies have shown that the potential toxicity of DEHP accelerates reproductive aging, causing decreases in ovarian follicle populations, oocyte quality, and embryonic developmental competence, and inducing pregnancy loss (Brehm et al., 2018; Chiang et al., 2020; Hannon et al., 2016; Pocar et al., 2017). *C. elegans* oocyte quality declines as a function of maternal age, regardless of oocyte number (Luo and Murphy, 2011). Additionally, studies indicate that the fertilizability of oocytes becomes compromised with age, since older worms are more likely to have a cluster of unfertilized oocytes in the uterus (Luo et al., 2010). DEHP possibly promotes reproductive aging by decreasing oocyte quality and numbers, which causes a decline in the brood size and egg hatchability.

All the observations imply that DEHP may induce reproductive aging and impose reproductive restrictions. Previous studies have showed the potential reproductive toxicity of DEHP (Hannon et al., 2016; Pocar et al., 2017; Ye et al., 2014). From one aspect, 72-hr exposure to DEHP evidently decreases the total germline cells and germline area, which may affect the total brood size (Figs. 1 and 2). From another, the increased expression of *unc-86* could significantly result in egg-laying defects in adults (Figs. 1 and 3). Therefore, we provide evidence to explain the reduction of reproductive capacity due to DEHP.

We put particular emphasis on the underlying mechanisms explaining the toxicity of DEHP in gonad development and reproductive capacity. One of the underlying mechanisms found in our research is the induction of increased autophagy. There is a close interplay between autophagy and the processes of cell death, proliferation and differentiation (Di Bartolomeo et al., 2010), and DEHP could induce more autophagy in worms by affecting the expression of *atg-18, atg-7, unc-51, bec-1* and *lgg-1*, which are core genes required for autophagy (Lapierre et al., 2013; Li et al., 2013; Zhang et al., 2015). Research has shown that *atg-18, atg-7* and *bec-1* are required for the late larval expansion of germline stem cell progenitors in the C. elegans gonad (Ames et al., 2017), suggesting that autophagy plays a critical role in the development of germline stem cells. Interestingly, not only insufficient but also excessive levels of autophagy lead to retarded cell growth in the nematode *C. elegans*, and genes *unc-51*/Atg1 and *bec-1*/*Atg6* are correlated to reduced cell size (Vellai et al., 2008). *Unc-51* and *bec-1* gene expression was significantly increased after DEHP exposure. Therefore, DEHP may induce smaller size in germline cells to decrease brood size and damage gonad development. Taken together, DEHP activated autophagy with increased autophagy-related gene expression and recognizable autophagosomes, which is consistent with a study on impairment of primordial folliculogenesis by DEHP (Zhang et al., 2018b). The study shows that DEHP induces autophagy by activating AMPK-SKP2-CARM1 signaling in mice perinatal ovaries, which results in disrupted primordial folliculogenesis and reduced female fertility (Zhang et al., 2018b). In *C. elegans*, AMPK activation though ULK1/2 complex induces autophagy by phosphorylating downstream autophagy complexes (Corona Velazquez and Jackson, 2018; Egan et al., 2011), indicating that autophagy is possibly induced by activating AMPK. Interestingly, DEHP and its metabolites can induce apoptosis through autophagy, indicating that DEHP-induced increases in autophagy can further increase cell damage (Li et al., 2019; Wu et al., 2017). DEHP possibly increased reproductive damage by increasing autophagy and ROS-induced cell apoptosis.

Another underlying mechanism is the induction of ROS in worms. Existing evidence suggests that ROS elevation is one of the key factors leading to reproductive injury; increased levels of ROS are possibly responsible for the increment of apoptosis and primordial follicle assembly impairment (Liu et al., 2019). Among the key endogenous antioxidants is SOD, which protects cells from ROS-mediated damage (Van Raamsdonk and Hekimi, 2010). Similarly, the glutathione peroxidase family is a major antioxidant enzyme family, which catalyzes the reduction of a variety of hydroperoxides to reduce oxidative damage (Sakamoto et al., 2014). Cells showed elevated levels of ROS, causing severe damage to cellular components such as DNA, membrane lipids and proteins, which can potentially lead to cell death (Arts et al., 2015; Kutuk et al., 2017). DEHP exposure increased the levels of ROS in *C. elegans*, implying that DEHP likely induced cell damage and caused insufficient gonad development, damaging the reproductive result.

DEHP exposure is associated with endocrine-related diseases in women of reproductive age. A previous study shows that DEHP increased ROS generation, which may be associated with the development of endocrine-related diseases such as endometriosis (Cho et al., 2015). DEHP significantly increased the ROS, GPX and SOD, indicating that SOD and GPX activities play an important role in defense against ROS damage. DEHP inhibited oocyte maturation, induced apoptosis and ROS and ATP increase in cumulus cells in horses, and at low doses (0.12 μmol/L) negatively influenced the ability of equine oocytes to reach meiotic maturation at the MII stage (Ambruosi et al., 2011). Low concentrations of DEHP significantly decreased the total germline cells and germline area, suggesting that DEHP may impact oocyte development to affect oocyte quality and then reduce fertility. In addition, research indicated that selenium supplementation effectively improved DEHP-induced disruption of redox equilibrium, suggesting that modulation of the redox status can reduce DEHP-induced damage (Zhang et al., 2018a). Our data suggest that GPX and SOD have a protective effect on DEHP-induced reproductive damage.

4. Conclusions

In conclusion, we employed the animal model *C. elegans* to determine the effect of DEHP on reproductive toxicity. In nematodes, DEHP could induce reproductive toxicity toward both gonad development and reproductive capacity. Moreover, we found that the DEHP could effectively enhance reproductive toxicity by increased autophagy and ROS in exposed nematodes. Induction of egg-laying defects, over-level expression of *unc-86*, autophagy and ROS may contribute greatly to the observed enhancement in DEHP toxicity in reducing reproductive capacity. Additionally, lower concentrations of DEHP promoted autophagy while higher concentrations inhibited autophagy. Lower concentration DEHP exposure over a long time period may lead to more severe reproductive damage. Also, SOD and GPX have a synergistic effect against ROS damage induced by DEHP. Our results imply the potential for DEHP to induce at least two aspects of reproductive toxicity in environmental organisms.

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