**Effects of 2-ethylhexyl diphenyl phosphate (EHDPP) on glycolipid metabolism in male adult zebrafish revealed by targeted lipidomic analyses**

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**Abstract:**

The present study aims to evaluate the effects of 2-ethylhexyldiphenyl phosphate (EHDPP) on glycolipid metabolism *in vivo*. Adult male zebrafish were exposed to various concentrations (0, 1, 10, 100 and 250 μg/L) of EHDPP for 28 days, and changes in lipid and glucose levels were measured. Results indicated significant liver damages in the 100 and 250 μg/L EHDPP groups, which both exhibited significant decreases in hepatic somatic index (HSI), elevated activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum and liver, as well as hepatocyte vacuolation and nuclear pyknosis. Exposure to 100 and 250 μg/L EHDPP led to significant reductions in serum and liver cholesterol (TC), triglycerides (TGs), and lipid droplet deposition, indicating a significant inhibition of EHDPP on hepatic lipid accumulation. Lipidomic analyses manifested that 250 μg/L EHDPP reduced the levels of 103 lipid metabolites which belong to glycerides (TGs, diglycerides, and monoglycerides), fatty acyles (fatty acids), sterol lipids (cholesterol, bile acids), sphingolipids, and glycerophospholipids, and downregulated genes involved in *de novo* synthesis of fatty acids (*fas*, *acc*, *srebp1*, and *dagt2*), while upregulated genes involved in fatty acid β-oxidation (*pparα* and *cpt1*). KEGG analyses revealed that EHDPP significantly disrupted glycerolipid metabolism, steroid biosynthesis and fatty acid biosynthesis pathways. Collectively, the results showed that EHDPP induced lipid reduction in zebrafish liver, possibly through inhibiting lipid synthesis and disrupting glycerolipid metabolism. Our findings provide a theoretical basis for evaluating the ecological hazards and health effects of EHDPP on glycolipid metabolism.

**Keywords:** EHDPP; lipid metabolism; lipid acids; zebrafish (*Danio rerio*); lipidomics

1. **Introduction**

2-ethylhexyl diphenyl phosphate (EHDPP) is a typical chemical within organophosphate flame retardants (OPFRs), which have been extensively utilized to improve the fire safety, durability and flexibility of a diverse range of products (van der Veen and de Boer, 2012). EHDPP is easily released from the products into the environment and can be ubiquitously detected in environmental matrices, including dust, surface water, and organisms (Salamova et al., 2014). For instance, the concentrations of EHDPP reached up to 480 ng/L in surface water samples from China (Liang et al., 2022) and 730 ng/L in Uganda (Nantaba et al., 2021). It was also reported that concentrations of EHDPP in wild fish ranged from 85 ng/g to 14, 000 ng/g as per lipid weight in Swedish, Norway and the Philippines (Hallanger et al., 2015; Kim et al., 2011; Sundkvist et al., 2010). Remarkably, consistent concentrations of EHDPP and related metabolites have been detected in samples of human blood and urine (Li et al., 2019; Liao et al., 2023). Notably, EHDPP was the most predominant OPFR in human whole blood in Chengdu, China, with a median concentration of 0.674 ng/mL (Guo et al., 2023). Considering the worldwide detection and high bioaccumulation of EHDPP, increasing concerns have been raised regarding the potential toxicities of this chemical.

Previous studies have shown that EHDPP was prone to induce thyroid dysfunction (Shu et al., 2024), developmental issues (Li et al., 2021; Negi et al., 2021; Yan et al., 2020), endocrine toxicity (Shen et al., 2019), and reproductive disorders (Yang et al., 2022a) among diverse organisms. Recentstudies conducted *in vitro* have underscored the lipotoxicity caused by EHDPP.Specifically, Sun et al (2020) discovered that EHDPP promoted adipogenesis in 3T3-L1 cells in mouse by activating peroxisome proliferator-activated receptor-γ (PPARγ). Exposure of human liver cells (HepG2) to EHDPP has been demonstrated to lead to lipid accumulation via nuclear receptors PXR and/or PPARγ (Negi et al., 2021). Using human 3D hepatospheroid cell, Negi et al (2023) found EHDPP activated PCG1α and increased TG levels. An epidemiological study showed that TG and total cholesterol (TC) were associated with the urinary levels of EHDPP and 5-OH-EHDPP (Zhao et al., 2019). Although several studies have indicated that maternal exposure to EHDPP altered glucose tolerance and induced liver damage in mice F1 offspring (Yan et al., 2020), theprecise *in vivo* effects of EHDPP on glycolipid remain unclear.

The liver is a crucial target in regulating glucose and lipid metabolism (Zou and Wang, 2023). With a similar structure and metabolic function to mammalian liver, zebrafish has been suggested to be an ideal model for liver disease research (Goessling and Sadler, 2015; Gut et al., 2017). Additionally, the fish is also an important model to monitor and assess pollutants in aquatic ecosystems. Furthermore, high levels of EHDPP have been observed in both wild fish (Sundkvist et al., 2010) and laboratory fish livers (Li et al., 2020; Li et al., 2021). The present study used zebrafish to evaluate the effects of EHDPP of environmentally relevant concentrations on glycolipid metabolism as well as the underlying mechanisms. Metabolic effects were comprehensively evaluated through morphological, biochemical and transcriptional assays. Targeted lipidomic analyses were applied to determine alterations of lipids and their metabolite levels in zebrafish liver. The results will provide novel insights into EHDPP effects on glycolipid metabolism in fish.

**2. Materials and methods**

**2. 1. Reagents**

EHDPP (CAS No. 1241-94-7; with purity exceeding 90%) was procured from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). Additionally, dimethyl sulfoxide (DMSO; CAS No:2206-27-1; purity greater than 99%) was from Sigma-Aldrich (St. Louis, MO, USA). Commercial kits used for determining alanine aminotransferase (ALT), aspartate aminotransferase (AST), [total cholesterol](http://www.njjcbio.com/products.asp?id=2581) (TC), [triglyceride](http://www.njjcbio.com/products.asp?id=2579) (TG), and [total bile acid](http://www.njjcbio.com/products.asp?id=818) (TBA) were procured from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The SYBR®Green PCR kit, PrimeScript®RT reagent kit, and TRIzol reagent were all procured from TaKaRa (Dalian, Liaoning, China). Other reagents were of analytical grade.

**2.2. Experimental zebrafish and treatment**

Healthy adult zebrafish (wild type AB strain, aged 3 months) were acquired from the China Zebrafish Resource Center located in Wuhan, China. For adult female zebrafish, lipid metabolic pathways in liver may be impacted during the spawning cycles, therefore, male adult zebrafish were used in this study (Jin et al., 2008; Uren Webster et al., 2013). Male zebrafish were randomly allocated into 15 glass tanks, with each tank housing 45 fish in 45 L carbon-filtered tap water. Following 2-week acclimation, zebrafish were subjected to different concentrations of EHDPP ranging from 0 to 250 μg/L (0, 1, 10, 100 and 250 μg/L, each concentration replicated in three separate glass tanks) for 28 days. The lowest exposure concentration (1 μg/L) was chosen to resemble EHDPP concentrations in surface water in China (480 ng/L) (Liang et al., 2022). The highest exposure concentration (250 μg/L) was adopted based on a previous study which indicated endocrine disruption effects of EHDPP on male zebrafish under this concentration for 21 dyas (Yang et al., 2022). The intermediate concentrations were established by gradient ascent.

Both the control and exposure groups received the same concentration of DMSO (0.01% (v/v)). Prior to the substitution of the exposure solution, carbon-filtered tap water was aerated for more than 24 hours, and the exposure solutions were refreshed daily with the aerated water to sustain stable EHDPP concentrations. During the exposure period, a photoperiod cycle of 14 hours of light and 10 hours of darkness was implemented, and the temperature was consistently sustained at 28 ± 0.5℃. Zebrafish were fed twice a day with live brine shrimp. After 28-d exposure, the adults were anesthetized using MS-222. After the measurement of body length and weight, zebrafish were dissected humanely to obtain blood and liver tissues for following analyses. All experiments adhered to the ethical guidelines expounded by the Institutional Animal and Care and Use Committees of Huazhong Agricultural University, under the approval number of HZAUFI-2018-020.

**2.3. EHDPP qualification in tissues and exposure solutions**

EHDPP concentrations in livers of zebrafish and exposure solutions were determined in accordance with our previous study (2024). For liver analyses, three livers in each replicate tank (n=3) were combined into one sample. This mixed samples were then freeze-dried, precisely weighed, ground into fine powder, and stored at -80℃ until subsequent analysis. 2 mL water sampled immediately after (T0) and prior to (T24) water replacement from each replicate tank (n=3) was used for exposure solutions quantification. The concentrations of EHDPP were quantified using the Waters® ACQUITY UPLC H-Plus Class system paired with the XevoTM TQ-XS mass spectrometer (Milford, USA). The limit of detection (LOD) for EHDPP stood at 0.05 μg/L. Recovery rates of EHDPP varied between 69.57% to 73.78% in exposure solutions and 70.80% to 76.33% in liver tissues. Detailed information on preparation of samples, procedures of analyses, and the quality assurance/quality control is provided in the Supplementary Materials (Text A1).

**2.4. Histological examination of livers**

After EHDPP exposure for 28 days, six livers from each replicate tank were fixed overnight in 4% paraformaldehyde for histological examination. Among these, three livers were used for hematoxylin & eosin (H&E) staining, while another three livers underwent Oil Red O staining. The livers were sliced into sections of 3-4 μm thickness before subjecting to staining of hematoxylin & eosin (H&E). For Oil Red O staining, frozen sections of 8-10 μm thickness were prepared from livers. The relative areas of lipid droplets were quantified by the ImageJ (v1.52) software (NIH, Tokyo, Japan).

**2.5. Biochemical indicator analysis**

The activities of ALT and AST, as well as contents of TG and TC in serum were determined using respective commercial kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Approximately 4-5 microliters of serum collected from three fish in the same tank were pooled as one sample (n= 3 replicates), and the activities of AST and ALT were evaluated after a 3-fold dilution with 0.9% saline solution. To determine the TG and TC contents, approximately 8-10 microliters of serum collected from six fish were pooled as a single sample (n=3 replicates) and evaluated following a 5-fold dilution with 0.9% saline solution.

Meanwhile, the activities of ALT and AST, as well as the contents of TC, TG, FFA, and BA were accurately determined in livers. Livers from three individual zebrafish in each tank were consolidated into one sample (n=3 replicates) and then weighed. After homogenization with 0.9% sodium chloride solution on ice, the supernatant was collected through centrifugation under 2500 rpm at 4°C for 10 minutes, and then used for quantification of protein and biochemical indicators mentioned above.

**2.6. Gene transcription**

Procedures on RNA extraction, isolation, and purification, as well as first-strand cDNA synthesis were performed according to our previous study (Cheng et al., 2017b). In brief, three livers from each treatment tank were sampled for RNA extraction. The primer sequences and amplifying procedures are listed in Table A1 and Text A2, respectively.

**2.7. Quantitative** **lipidomic analysis**

Three livers collected from three individuals were combined into one sample (n=6 replicates). Lipid extraction was carried out following the protocol outlined in the Supplementary Materials (Text A3). Targeted lipidomic analyses were conducted using the AB Sciex QTRAP 6500 liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform. Qualitative lipidomic analyses were performed using Metware database (<http://www.metware.cn/>). etails on statistical analyses are provided in Supplementary Materials (Text A3).

**2.8. Statistical Analysis**

Data analyses and graphical representation were performed using GraphPad Prism (v.8.0.2, GraphPad Software). Prior to any statistical comparisons, the normality and homoscedasticity of all data were rigorously assessed. Differences between EHDPP treatment groups and the control group were determined via one-way analysis of variance followed by Dunnett's multiple comparison test. Any differences yielding a p-value smaller than 0.05 were considered to be statistically significant.

**3. Results and Discussion**

**3.1. Concentrations of EHDPP in exposure solutions and zebrafish liver**

During the exposure period, EHDPP concentrations were determined to be largely different between the newly prepared medium and the water samples collected after 24 h fish culturing (Table 1). Besides the natural degradation on EHDPP in water environment (Cristale et al., 2017), the bioaccumulation of this chemical may be a main factor to explain this phenomenon (Li et al., 2021). Indeed, high residues of EHDPP were observed in the liver, with contents of 245.51 ± 0.10, 1 846.66 ± 1.03, 7722.60 ± 3.76, 37 190.24 ± 15.41 ng/g lw, respectively. Similar prominent bioaccumulations were also observed in Japanese medaka liver (Li et al., 2020) and zebrafish larvae (Shu et al., 2024) and has been attributed to the high lipophilicity of EHDPP (log Kow =5.73).

Table 1. Concentration of EHDPP in exposure solution and livera

|  |  |  |  |
| --- | --- | --- | --- |
| Nominal concentration（μg/L） | Measured concentration（μg/L） | | Concentration in the liver (ng/g lw) |
| T0 | T24 |
| 0 | ND | ND | ND |
| 1 | 1.15 ± 0.17 | 0.13 ± 0.40 | 244.51 ± 0.10 |
| 10 | 12.67 ± 3.08 | 1.03 ± 0.13 | 1845.66 ± 1.03 |
| 100 | 77.14 ± 19.90 | 15.72 ± 3.45 | 7722.60 ± 3.76 |
| 250 | 159.92 ± 19.95 | 47.72 ± 9.43 | 37190.24 ± 15.41 |

a ND: not detected. Data are presented as mean ± SEM. Each group contains 3 replicates (n=3).

**3.2. Liver impairments in zebrafish after exposure to EHDPP**

During the 4-week exposure, EHDPP did not alter the survival rate, body length and body weight of adult male zebrafish. However, upon exposure to EHDPP of 10, 100, and 250 μg/L, the HSI values of zebrafish were significantly decreased by 11.01%, 10.53%, and 10.01%, respectively, compared with the control group (Table 2), implying that liver impairments had occurred. HSI is a commonly used indicator to assess liver health in fish. Although few studies have reported decreased HSI induced by EHDPP in fish, similarly phenomenon was observed with other OPFRs (e.g., TDCIPP) (Sun et al., 2023). Yang et al (2022b) found that HSI values of 7-day-old male chickens were increased by EHDPP (1600 or 3200 mg/kg body weight). The inconsistency between the results of this study and our present study might be attributed to different exposure concentrations and modes. However, all these results manifested that EHDPP has the potential to affect liver health in organisms.

In addition to HSI index, histopathological characters of liver were examined by hematoxylin-eosin staining in our results. No significant differences were found between the lower EHDPP concentration (1, 10, 100 μg/L) groups and the control group, however, hepatic cytoplasmic vacuolization and pyknotic nucleus were found in the highest EHDPP exposure group (250 μg/L) (Fig. 1A). Although it is the first time that the microstructure damage induced by EHDPP has been reported in adult zebrafish liver, pathological changes including vacuolar degeneration and disappearance of nuclei in hepatocytes have previously been described in 7-day-old male chickens orally given EHDPP (1600 or 3200 mg/kg body weight) for 42 days (Yang et al., 2022b). In addition, ALT and AST are the major indicators of hepatic dysfunction (Amacher, 2002). In this present study, ALT activities in liver were increased in the 250 μg/L EHDPP exposure group (Fig.1B and C). Similarly, a significant increase in serum AST activity was observed in the 250 μg/L EHDPP group compared with the control group (Fig.1D and E). Previous studies on zebrafish have also documented a significant elevation in AST and ALT activities resulted from triphenyl phosphate (TPhP) treatment (Ramesh et al., 2020). Collectively, these findings validate the liver damage in male zebrafish induced by EHDPP.

Table 2. Developmental parameters in male adult zebrafish after EHDPP exposure (0, 1, 10, 100 and 250 μg/L) for 28 daysa

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| EHDPP (μg/L) | Survival rate (%) | Body weight (g) | Body length (mm) | Body mass index (kg/m2) | Hepatic somatic index（%） |
| 0 | 93.33 ± 1.81 | 0.47 ± 0.05 | 29.93 ± 1.14 | 0.53 ± 0.06 | 2.72 ± 0.80 |
| 1 | 95.56 ± 1.81 | 0.46 ± 0.05 | 29.70 ± 1.12 | 0.52 ± 0.06 | 2.69 ± 0.91 |
| 10 | 93.33 ± 1.15 | 0.47 ± 0.05 | 29.76 ± 1.14 | 0.54 ± 0.06 | 2.32 ± 0.66\*\*\* |
| 100 | 93.33 ± 1.15 | 0.48 ± 0.06 | 30.23 ± 1.38 | 0.53 ± 0.07 | 2.33 ± 0.57\*\*\* |
| 250 | 92.56 ± 1.85 | 0.47 ± 0.05 | 30.08 ± 1.07 | 0.52 ± 0.08 | 2.35 ± 0.55\*\*\* |

a Values were expressed as mean ± SEM of three replicates. \*\*\* *p* < 0.001 indicates significant difference between EHDPP exposure groups and the control group.

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Figure 1. Liver histology of male adult zebrafish after exposure to EHDPP for 28 days. A, representative liver sections by H&E staining. Green arrowhead indicates hepatic cell vacuolar degeneration, while yellow triangle indicates pyknotic nucleus. B and C, hepatic AST and ALT activities, respectively. D and E, serum AST and ALT activities, respectively. Data are expressed as mean ± SEM of three replicates. *\*P* < 0.05and *\*\*\*P* < 0.001 indicate significant differences between exposure groups and the control group.

**3.3. EHDPP effects on lipid metabolism in liver**

In comparison to the control group, the relative areas of lipid droplets were significantly decreased in the 100 and 250 μg/L EHDPP exposure groups (Fig. 2A). Consistent with this result, a newly published study showed that lipid accumulation content in the liver was significantly decreased by 200 μg/L EHDPP exposure in zebrafish larvae (Xu et al., 2023). However, several *in vitro* studies showed that EHDPP promoted lipid storage in HepG2 (Negi et al., 2021) and 3T3-L1 preadipocytes cells (Sun et al., 2020). These results confirmed that EHDPP disturbs the hepatic lipid metabolism, yet noteworthy differences exist between the *in vivo* and *in vitro* models.

Furthermore, when compared to the control group, lipid parameters including TG and TC contents in the 100 and 250 μg/L exposure groups were remarkedly decreased. The decreased TG and TC contents in the serum (Fig. 2B and C) and livers (Fig. 3A and B) manifested that EHDPP inhibited the lipid accumulation in male zebrafish. Consistently, Xu et al. (2023) also found that a 7-day EHDPP exposure at 100 μg/L resulted in decreased TG levels in zebrafish larvae. Perinatal exposure to EHDPP notably decreased the TG contents of male mouse offspring (Yan et al., 2020). On the other hand, a newly published study found no significant differences in TG and TC contents of adult zebrafish (mixed male and female) after a 21-day EHDPP exposure even at the highest concentration (245 μg/L) (Yang et al., 2023). A possible explanation for these different outcomes might be a male-biased disruptive effect on hepatic lipid metabolism induced by EHDPP. In order to explore the possible mechanism for the decreased TG levels, we measured the contents of free fatty acids (FFAs) which are the major sources to the synthesis of TG (Fukuda and Ontko, 1984). The current study observed a significant reduction in FFA levels, specifically, a 46.93% decrease in the 250 μg/L exposure group when compared to the control group (Fig. 3D). This change may be partially due to the decreased FA synthesis induced by decreased gene transcriptions of fatty acid synthetase (*fasn*) and cetyl-CoA carboxylase1 (*acc1*). Sterol regulatory element binding protein 1 (SREBP1) functions as a crucial transcription factor regulating expressions of lipogenic genes in fish and mammals, e.g., *acc* and *fas* (Shimano and Sato, 2017;Guan et al., 2019). Acetyl-CoA carboxylases alpha (acc1) is a pivotal rate-limiting enzyme in transformation from acetyl-CoA to malonyl-CoA, thereby plays an important role in the *de novo* synthesis pathway of FAs (Cronan and Waldrop 2002). FASN facilitates the conversion of acetyl-CoA and malonylCoA into palmitate, which is then esterified to form triglyceride (Knobloch et al., 2013). In our study, the down-regulation of the expressions of *srebp1c, fasn*, *acc1* indicated that exposure to EHDPP suppressed the *de novo* lipogenesis (Fig. 4; Fig. A1). Similarly, Yan et al. (2020) found that perinatal exposure to 30 and 300 μg/kg bw/day EHDPP resulted in decreased TG contents accompanied with down-regulated expression of *srebp1c* in F1 male offspring mice. Meanwhile, significant down-regulation of 3-hydroxy-3-methylglutaryl-CoA reductase (*hmgcra*) was found under EHDPP treatment compared to the control group. The HMG-CoA reductase (HMGCR) oversees the conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate, which is a crucial step in the cholesterol biosynthesis pathway (Chen et al., 2019). The down-regulation of gene transcript of *hmgcra* in the EHDPP group suggested that cholesterol synthesis might be inhibited in male zebrafish. A comparable observation was also documented by Aluru et al. (2021), which indicates that cholesterol biosynthesis genes in Atlantic Cod (*Gadus Morhua*) liver were significantly down-regulated after EHDPP treatment.

Additionally, EHDPP exposure upregulated the mRNA expressions of carnitine palmitoyl transferase 1 (*cpt1*) and peroxisome proliferators activated receptor α (*pparα*) (Fig. 4), implying that EHDPP might promote lipolysis. *Pparα* is expressed primarily in the liver, and plays a crucial role in mitochondrial β oxidation, as well as in regulating fatty acid catabolism (França et al., 2019; Montagner et al., 2016). In alignment with our findings, Xu et al. (2023) likewise reported that a 7-day EHDPP exposure at 200 μg/L resulted in increased *pparα* levels in zebrafish larvae. Cpt1 is responsible for encoding a rate-limiting enzyme that governs the β-oxidation process of fatty acid (Wang et al., 2022). The upregulation of transcript levels of *pparα* and *cpt1* in our study might suggest that EHDPP could promote β-oxidation of fatty acid in the male zebrafish liver. Previous studies have reported potential effects on β-oxidation process by other OPFRs. For example, Liu et al. (2022) have reported that TPhP (another kind of OPFRs) increased transcript levels of *pparα* and *cpt1* in male mice.

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Figure 2. Effects of EHDPP exposure on lipid concentrations in male adult zebrafish. (A) Representative liver sections by Oil Red O staining and relative area of lipid droplets (B) serum TC levels, and (C) serum TG levels. The black arrow indicates lipid droplets. Data are expressed as mean ± SEM of three replicates. *\*P <* 0.05, *\*\*P <* 0.01, and *\*\*\*P <* 0.001 indicate significant differences between the exposure groups and the control group.

Figure 3. Lipid contents of TG (A), TC(B), BA (B), and FFA (D) in male zebrafish liver after exposure to various concentrations of EHDPP for 28 days. Values are presented as means ± SEMs (n = 3).*\*P <* 0.05indicates significant differences between the exposure groups and the control group.



Figure 4. Effects of EHDPP on genes involved in lipid metabolism in male zebrafish liver. Values are presented as means ± SEMs (n = 3). *\*P <* 0.05, *\*\*P <* 0.01, and *\*\*\*P <* 0.001 indicate significant differences between the exposure groups and the control group.

**3.4. EHDPP effects on glymetabolism**

Considering that fatty acid is synthesized from acetyl CoA which mainly comes from pyruvate produced by glycolysis (Zhao et al., 2021), we evaluated the potential effects of EHDPP on glycolysis. As shown in Fig. 5A, exposure to 100 and 250 μg/L EHDPP significantly elevated hepatic glucose levels, while the pyruvate content exhibited a decline trend in these groups (Fig. 5B). Gene expressions of hexokinase (*hk*), glucokinase (*gk*), and pyruvate kinase (*pk*) were significantly decreased in the 100 and 250 μg/L EHDPP groups, suggesting significant EHDPP inhibition on glycolysis (Fig. 5C). PK is a key enzyme in the glycolysis process, which catalyzes the conversion of phosphoenolpyruvate to pyruvate (Isidor et al., 2020). HK phosphorylates sugar immediately upon entering cells, enabling subsequent metabolism of these sugars (Irwin and Tan, 2008). In line with our findings, Xu et al. (2023) have also discovered that a 7-day exposure to EHDPP resulted in decreased *pk* levels in zebrafish larvae. Glucokinase is the main glucose-phosphorylating enzyme in hepatocytes, which catalyzes the first reaction in glucose metabolism (Agius, 1998; de la Iglesia et al., 2000). In this study, inhibition on *pk, gk* and *hk* gene expressions confirmed that EHDPP could cause glycometabolic disorders by inhibiting glycolysis.



Figure 5. Effects of EHDPP on levels of glucose (A) and pyruvate acid (B) and transcriptions of mRNA related to glucose metabolism (C) in male zebrafish liver. Values are presented as means ± SEMs (n = 3). *\*P <* 0.05*,* and *\*\*P <* 0.01 indicate significant differences between the exposure groups and the control group.

**3.5. EHDPP effects on lipid metabolite in zebrafish liver**

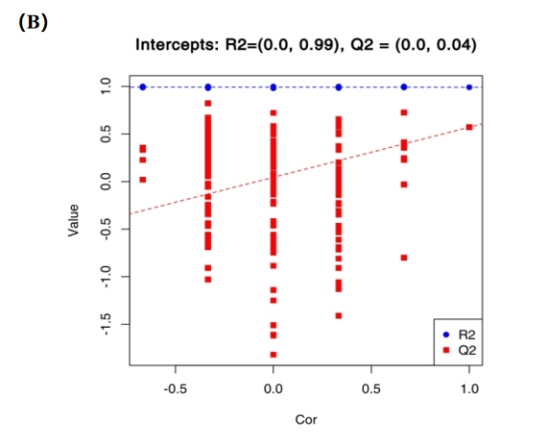
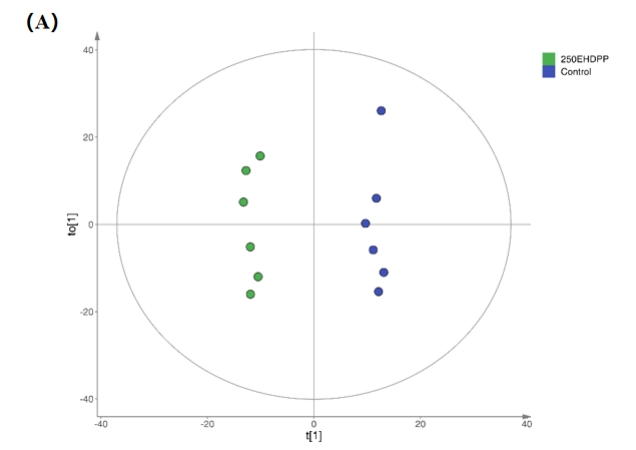
Targeted lipidomic analyses were undertaken to reveal variations in lipidome between the 250 μg/L treatment group and the control group. As presented in Table S2, the lipidomic analyses identified 886 lipid species which belong to six lipid categories, including 389 glycerolipids (GLs), 222 glycerophospholipids (GPs), 141 sphingolipids (SPs), 18 sterol lipids (STs), 2 prenol lipids (PRs), and 114 fatty acyls (FAs). In total, 43 different lipid classes were identified between the control group and the 250 μg/L exposure group, among which TGs were the most abundant (360 lipid species), followed closely by acyl carnitine (CAR) and ceramide (Cer-NS) (47 and 45 lipid species, respectively) (Fig. A1). The orthogonal partial least squares discriminant analysis (OPLS-DA) model suggested distinct patterns between the 250 μg/L EHDPP exposure group and the control group (Fig. 6A). Among all model groups, categorical variables R2 exceeded 90%, indicating stability of the models. Notably, the intercept of the Q2 regression line stood at 0.04, which is below 0.05, thus suggesting that the OPLS-DA model did not exhibit overfitting (Fig. 6B). As shown in Fig. 6C, differentially expressed lipids (DELs) were filtered and identified based on their variable importance in projection (VIP) scores. Specifically, 104 differentially altered lipid metabolites were identified, with a single metabolite displaying upregulation, while the remaining 103 metabolites exhibiting significant downregulation. The separation of the control and EHDPP group was mainly driven by alterations of GLs, GPs, SPs, STs, PRs, and FAs. As depicted in Fig. 6D, the lipidomic analyses revealed that the alterations in liver lipid compositions induced by EHDPP were characterized by variations of 40 triglycerides (TGs), 13 diglycerides (DGs), 4 monoglyceride (MGs), 1 diglyceride [ether](https://cn.bing.com/dict/search?q=ether&FORM=BDVSP6&cc=cn) [linkage](https://cn.bing.com/dict/search?q=linkage&FORM=BDVSP6&cc=cn) (DG-O), 1 phosphatidylethanolamine (PE), 1 phosphatidylcholine (PC), 2 lyso-phosphatidylcholine O (LPC-O), 1 lyso-phosphatidylethanolamine (LPE-P), 1 coenzyme Q (COQ), 15 free fat acids (FFAs), 7 eicosanoids, 1 acyl carnitine (CAR), 5 cholesteryl esters (CEs), 4 bile acid (BA), 2 ceramides-ADS (Cer-ADS), 2 ceramides-NS (Cer-NS), 2 glycosphingolipids (HexCer-NS), and 2 sphingomyelins (SMs) (Figure. 6D (a) and (e)).

Glycerolipids, including TG, DG and MG, are the predominant forms of cellular energy storage (van Meer et al., 2008). In the present study, lipidomic analyses confirmed that TG levels were significantly decreased by EHDPP exposure, which is in consistent with the results of the TG contents in serum and liver. Among the 360 species of TGs identified, 40 species exhibited significant decreases, with the change ratios reaching up to -11.11% (Table S3). As depicted in Figure D (b), the top 15 lipid species ranked based on fold change include TG (18:1\_22:5\_22:6), TG (19:0\_18:1\_22:6), TG (15:0\_18:1\_22:1), TG (18:1\_20:1\_24:5), TG (18:0\_18:1\_22:3), TG (18:0\_22:5\_22:6), TG (10:0\_16:0\_18:3), and TG (20:0\_18:1\_22:6). In addition to TG, the contents of 13 DGs and 4 MGs were also significantly decreased in the current study. As is well-known, MGs and DGs served as key intermediate products in the production of TGs through the glyceride monoester pathway (Wakil and Abu-Elheiga, 2009). Therefore, we deduced that the lipid synthesis and metabolism were disrupted by EHDPP. The enrichment analyses of the KEGG pathway also revealed that exposure to EHDPP had a significant impact on glycerolipid metabolism. A previous study on cresyl diphenyl phosphate also reported the potential of OPFRs to decrease glycerolipid contents in zebrafish embryos (Jin et al., 2024).

Free fatty acids (FFAs) are a class of organic acids that function as important energy-generating nutrients (Hara et al., 2014). As depicted in Fig.6D(f), lipidomic analyses revealed that 15 kinds of FFAs were dramatically inhibited by 250 μg/L EHDPP treatment, which is consistent with the results of FFA contents in liver detected by ELISA. All these results indicated that lipid homeostasis and energy metabolism were disrupted after EHDPP exposure. Linolenic acid (FFA (18:3)) and linoleic acid (FFA (18:2)) are essential fatty acids in organisms, which can be only derived from food and metabolized by β-oxidation (Hara et al., 2013). The downregulation of FFA (18:2) and FFA (18:3) indicated either reduced absorption or increased expenditure of these essential fatty acids in zebrafish after EHDPP treatment, potentially through an enhanced fatty acid β-oxidation process. Gene expressions related to fatty acid β-oxidation (*pparα* and *cpt1*) were indeed upregulated, suggesting that EHDPP could promote β-oxidation of fatty acid in the male zebrafish liver. In this study, palmitoleic acid (FFA (16:1)) contents were significantly inhibited by EHDPP. FFA (16:1) is synthesized by adding a double bond to palmitic acid which represents the major product of fatty acid synthase in the liver (Price, 2010). On the other hand, the expressions of genes (*srebp1c, fasn*,and *acc1*) related to *de novo* FA synthesis were dramatically inhibited in our study. Therefore, the FFA (16:1) decrease might be caused by the decrease in *de novo* FA synthesis.

Sphingolipids serve as structural components of membranes and signaling molecules (Merrill, 2011). In the present study, EHDPP treatment significantly impacted sphingolipid metabolism. Sphingolipids, including CER (Cer (d18:0/20:0(2OH)), Cer (d18:0/22:1(2OH)), Cer (d17:1/21:0), Cer (d17:1/18:0)), and further metabolites of CER, such as SM (SM (d18:1/19:1), SM (d18:0/14:0)), HexCer (HexCer (d16:1/24:1), and HexCer(d16:1/22:0)), were significantly decreased by EHDPP (Fig. 6D (g)). Similarly, perturbations in total CER as well as HexCer and SM have also been reported in human 3D hepatospheroid cells in response to EHDPP (Negi et al., 2023). A previous research has established a connection between diminished sphingolipid metabolism and the pathogenesis of type 2 diabetes in human beings, as well as impaired function of pancreatic β-cell in mouse models (Khan et al., 2020). Indeed, increased glucose contents accompanied by significant decrease of expressions of genes related to glycolysis were observed in our study. Therefore, defects mediated by EHDPP in the pathway of sphingolipid metabolism might suggest a potential risk for diabetes development (Negi et al., 2023).

In the current study, lipidomic analyses have revealed a marked decline in the contents of sterol lipids, including BA (ursocholic acid, lithocholicacid-3-sulfate, glycocholic acid, and taurocholic acid) and cholesterol (CE (18:2), CE (20:3), CE (18:4), CE (22:3), and CE (26:6)) (Figure. 6D (h)). These results were consistent with substantial reduction in total cholesterol and total BA levels detected by ELISA assay. Similarly, in a previous study, a decrease in cholesteryl linoleate CE (18:2) was also noted in human 3D hepatospheroid cell, possibly due to the increased oxidation of CEs (Negi et al., 2023). Cholesterol serves as the precursor for the biosynthesis of various steroids, including bile acids (BA), sex hormones, and corticosteroids (Wang et al., 2017). The decrease in BA contents could be potentially attributed to the cholesterol decrease, which indicated disturbed bile acid/cholesterol metabolism in zebrafish in response to EHDPP exposure. Similarly, EHDPP-induced alterations in bile acids/cholesterol homeostasis have been documented in chicken embryonic hepatocytes at the gene transcription level (Shen et al., 2019). In the current study, the KEGG classification further validated that EHDPP exerts an influence on steroid biosynthesis as well as taurine and hypotaurine metabolism (Fig.6E).



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Figure 6. Metabolomic alterations in male zebrafish liver after EHDPP exposure for 28 days. (A) OPLS-DA score plot of liver metabolites. (B) Permutation test of the OPLS-DA model. (C) Lipid volcano map depiction for the 250 μg/L EHDPP group. (D) Number of sub-types of GLs, GPs, and PRs (a), as well as FAs, STs, and SPs (e) upon EHDPP exposure; and representative GLs (b), GPs (c), PRs (d), FAs (f), STs (g), and SPs (h) of different concentrations. In each row, the box on the left represents 0 μg/L EHDPP, while the box on the right represents 250 μg/L EHDPP. Fold changes are shown in different colors (red: increased; green: decreased). (E) KEGG pathway enrichment analyses based on the significantly changed metabolites.

**4. Conclusion**

In conclusion, EHDPP exposure disrupted glycolipid metabolism in male zebrafish, including liver impairments, lipid droplet deposition, reduction in TG, TC, BA, and FFA levels, and increase in glucose. Lipidomic analyses showed downregulation of major lipid classes after 250 μg/L EHDPP treatment, along with altered expressions of genes involved in *de novo* synthesis of FFAs, fatty acid β-oxidation, and glycolysis(Fig. 7). These results indicated that EHDPP reduced lipids in zebrafish liver by inhibiting biosynthesis and disrupting glycerolipid metabolism. Given EHDPP’s prevalence, the effects of long-term exposure to EHDPP on glycolipid metabolism should be investigated.

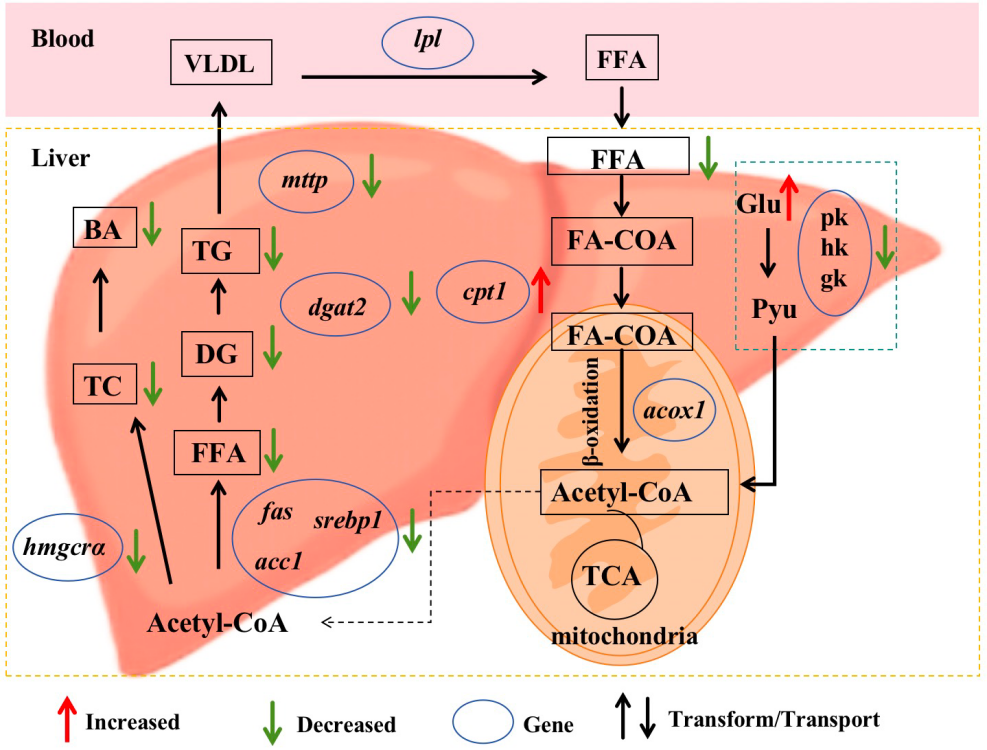


Figure 7. Schematic representation depicting the perturbed glycolipid metabolic pathways after exposure to EHDPP. Abbreviations: DG, diacylglycerol; *dgat2*, diacylglycerol acyltransferase 2 gene; TG, triacylglycerol; *mttp*, microsomal triglyceride transfer protein gene; FFA, free fatty acid; BA, bile acid; TC, total cholesterol; *srebp1*, sterol regulatory element binding protein 1 gene; *fas*, fatty acid synthetase; *acc1*, cetyl-CoA carboxylase1 gene; VLDL, very low density lipoprotein; FA-CoA, fatty acyl-CoA; *cpt1*, carnitine palmitoyltransferase 1 gene; *acox1*, acetyl-CoA oxidase 1 gene; TCA, tricarboxylic acid; Glu, glucose; Pyu, pyruvic acid; *hk*, hexokinase gene; *gk*, glucokinase gene; and *pk*, pyruvate kinase.

**Conflicts of interest**

The authors have no conflicts of interest to declare.

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**Appendix A. Supplementary Data**

Text A1. Quantification procedure and Quality Assurance and Quality Control (QA/QC). Text A2. Details of gene expression. Text A3. Details of quantitative lipidomic analysis. Table A1. Primer sequences used for qRT-PCR. Table A2. Hepatic lipid composition in the control and 250 μg/L EHDPP groups. Table A3. The top 40 lipids with differential changes. Figure A1. Effects of EHDPP on gene transcripts in male zebrafish liver. Figure A2. Lipid subclasses identified in male zebrafish liver.

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