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In vivo and *in vitro* toxicities of diethyl phthalate to flounder fish *Paralichthys olivaceus* and its gill cell line (FG cells)

Authors Info

Qin Xiao^{1*}, Daizong Li¹, Ran Guo¹, Lei Zheng², Xinlong An¹ and Zhaochun Zeng^{1*}

¹College of Ocean, Hebei Agricultural University, Qinhuangdao, 066 003, China

²College of Water Sciences, Beijing Normal University, Beijing, 100875, China

*Corresponding Author Email : qinxiao669@163.com

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Abstract

Aim : Diethyl phthalate (DEP) as a plasticizer and softener of daily supplies, is immensely released in the aquatic environment. The study was undertaken in order to evaluate the toxicity of DEP using the marine flounder (*Paralichthys olivaceus*) and its gill cell line (FG cells) *in vivo* and *in vitro*.

Methodology : *In vitro* toxicity of DEP to FG cells was determined by neutral red uptake assay and methyl thiazolyl tetrazolium assay, along with observing the change of cellular morphology by a phase contrast inverted microscope. *In vivo* toxicity of DEP to flounder fish was evaluated by calculating the median lethal concentration (LC₅₀) in semi-static condition, observing the histopathological change in liver and kidney of flounders by staining with hematoxylin and eosin, and determining the biochemical parameters using an AEROSSET automatic biochemical analyzer.

Results : DEP was acutely toxic to FG cells for 24hr in the range of 200 µg ml⁻¹-800 µg ml⁻¹. 50% inhibition of cell proliferation (IC₅₀) of DEP for 24 hr were 536.76 µg ml⁻¹ and 573.84 µg ml⁻¹ for NR uptake and MTT assay, respectively. Meanwhile, the FG cellular morphology was also negatively altered with DEP concentration increasing. In semi-static condition, the median lethal concentration (LC₅₀) of DEP for 96 hr was 31.92 µg ml⁻¹ for *Paralichthys olivaceus*. DEP also caused various extents of histological structures damages in livers and kidneys when fish were exposed to 5, 10 and 15 µg ml⁻¹ DEP, as evidenced by the necrosis of hepatocytes, the disorder of hepatic sinusoids, the degradation of tubular epithelial cells and multiple hemorrhages in kidneys. Meanwhile, the levels of alanine aminotransferase and lactate dehydrogenase were enhanced at the same concentration DEP treatments.

Interpretation : The study suggests that FG cell line can be a short term bioassay model for screening the potential toxicity of the pollutant, while living fish are a necessary model for studying DEP toxicity. This research will provide significant references for monitoring DEP pollution.

Introduction

Diethyl phthalate as a low molecular weight phthalate esters (PAEs), is extensively used as a plasticizer and softener in many products including automotive components, herbicides, toys, cosmetic formulations, medical treatment tubing, plastic packing films and so on (Page and Lacroix, 1995; Api, 2001). Since diethyl phthalate is not chemically bound to these polymer products, it can migrate readily from the plastics to the environment, especially the aquatic environment (Staples *et al.*, 2000). It is frequently detectable worldwide in surface water as well as drinking water, leading to a significant exposure of the compound to organisms (Leitz *et al.*, 2009). Its deleterious effects on human being have been reported, mainly interfering with the endocrine and immune systems (Giulivo *et al.*, 2016). Diethyl phthalate toxicity has recently become a great environmental concern in aquatic ecosystems. The evaluation for DEP toxicity is foremost and should be done first.

The study of diethyl phthalate toxicity has been continued nearly 20 years. Previously, its acute toxicity was mainly determined using rats, guinea pigs, rabbits and chickens, and its lethal doses are more than 1 g kg^{-1} via oral or peritoneal administration (Api, 2001). Afterwards, some mammal cell lines, e.g. human breast carcinoma cell line (MCF-7) and Chinese hamster ovary (CHO) cells, were also used to assess the impact of diethyl phthalate (Hong *et al.*, 2005; Kumar *et al.*, 2014, 2015). In aquatic environment, the diethyl phthalate toxicity is usually described by median lethal concentrations (LC_{50}) of fish, such as *Cyprinus carpio* (Barse *et al.*, 2007), *Cirrhina mrigala* (Ghorpade *et al.*, 2002) etc. In addition, fish (*Pimephales promelis*, *Danio rerio*) embryos have been also used to study diethyl phthalate toxicity (Mankidy *et al.*, 2013; Kim *et al.*, 2015). However, there are limited reports regarding the effect of diethyl phthalate toxicity on fish cell lines. Therefore, it is necessary to provide academic data about diethyl phthalate toxicity on fish cell lines.

The marine flounder, *Paralichthys olivaceus*, is an important cultured fish species in the Far East area including China, Japan and South Korea, has been successfully used to study the toxic effects of environmental pollutants (Nakayama *et al.*, 2008; Kang *et al.*, 2010; Vinay *et al.*, 2014). Gill cell line (FG cells) of *Paralichthys olivaceus*, established from gill tissue (Tong *et al.*, 1997), have also been widely applied in xenobiotic chemicals' cytotoxicity examinations (Xiao *et al.*, 2007, 2011; Na *et al.*, 2009; Pandey *et al.*, 2014). Thus, the present aimed to study *in vitro* toxicity of diethyl phthalate and *in vivo* toxicity marine founder fish, and as well as compare the strengths and weaknesses of two kinds of bioassay models.

Materials and Methods

FG cells : The FG cell line was derived from the gill tissues of flounder (*Paralichthys olivaceus*) and maintained following to the method of Tong *et al.* (1997). Briefly, the FG cells were cultured in

minimal Essential Medium (MEM; Gibco BRL, New York, USA) supplemented with 10% Bovine Calf Serum (BCS; Hyclone, Utah, USA), 100 IU ml^{-1} penicillin and 100 mg ml^{-1} streptomycin in plastic culture flasks (Corning, New York, USA) at 20°C .

Fish maintenance : Marine flounders (*Paralichthys olivaceus*) ($8.9 \pm 0.25 \text{ cm}$) were purchased from a local fish farm in Qinhuangdao, China. These fish were acclimatized in aerated natural seawater ($18\text{--}22^\circ\text{C}$) under ambient photo period in laboratory for at least 2 week before use. The fish were fed with live food twice a day.

DEP acute toxicity to FG cells *in vitro* : Cells were harvested and diluted to a concentration of $1.25 \times 10^5 \text{ cells ml}^{-1}$ in MEM with 10% BCS. The cell suspension was agitated and 200 μl aliquots were added to each well of 96-well tissue culture plates (Costar, Cambridge, MA, USA). Plates were incubated overnight at 20°C and next day the medium was removed. Based on the preliminary results, the cells were re-fed with medium containing 0 (solvent control), 100, 200, 400, 600 and 800 $\mu\text{g ml}^{-1}$ diethyl phthalate. Then two endpoints for toxicity, *i.e.*, NR uptake assay and MTT assay were determined after 24 hrs diethyl phthalate exposure. Furthermore, the morphological changes of FG cells were also observed and photographed every day using a phase contrast inverted microscope (Nikon Eclipse TS-100, Japan).

Neutral red uptake assay, as described by Borenfreund and Puerner (1985), measured inhibition of cell growth, which was based on the absorbance of vital dye NR by living, but not by dead, cells. After 24 hrs diethyl phthalate exposure, the test medium was replaced by a 200 μl medium containing 50 mg ml^{-1} neutral red, which had been pre-incubated overnight at 20°C and filtrated prior to use to remove fine precipitates of dye crystals. After *in situ* incubation for 3 hrs at 20°C , the cells were rinsed with

Fig. 1 : *In vitro* cytotoxicity of diethyl phthalate (0, 100, 200, 400, 600, 800 $\mu\text{g ml}^{-1}$) to FG cells as justified by NR assay and MTT assay. The individual data points are expressed as the arithmetic mean percentage of control $\pm$ S.E. ($n=3$)

Fig. 2 : Morphological changes of FG cells exposed to varied concentrations of diethyl phthalate for 24 hrs. Panels A through F correspond to 0(control), 100, 200, 400, 600 and 800 $\mu\text{g ml}^{-1}$ diethyl phthalate. Scale bars: 50 μm

warmed phosphate-buffered saline and then destained with 200 μl solution consisting of glacial acetic acid-96% ethanol-water (1:50:49). After rapid agitation for 10 mins at room temperature, the absorbance of the solution in each well was measured at 540 nm with a microplate reader (Unico, CA, USA) and the IC_{50} value

(concentration of test agent which causes a 50% inhibition in cell proliferations) was determined.

MTT assay described by Borenfreund *et al.* (1988) was based on inhibition by chemical injury of the reduction yellow MTT

Fig. 3 : Light micrographs of transverse section of the flounder (*Paralichthys olivaceus*) liver in 0 (control), 5, 10 and 15 $\mu\text{g ml}^{-1}$ diethyl phthalate exposure for 14 days (H&E staining). A: Control liver tissues. N: Hepatocyte nucleus; Bv: Blood vessel. B, C and D: Liver tissues of flounder exposed to 5, 10 and 15 $\mu\text{g ml}^{-1}$ diethyl phthalate, respectively. S: Sinusoid; Pn: Pycnotic nucleus; V: Vacuolation. Scale bars: 25 μm

tetrazolium salt to a blue insoluble MTT formazan product by the mitochondrial succinic dehydrogenase. After a 24 hrs exposure period, the test medium was replaced by PBS containing 20 μl 5 mg ml^{-1} MTT. After incubation for 4 hrs at 20°C, the solution was carefully removed and the cells were rinsed twice with rapidly. Then 150 μl per well dimethylsulfoxide was added to dissolve the purple formazan crystals that were produced. The absorbance of each well was measured at 490 nm and the IC_{50} value was calculated.

All experiments were performed at least three times, and the average absorbance at each dose was calculated and

expressed as percentage of absorbance of cells incubated with different doses of diethyl phthalate against control.

DEP acute toxicity to flounders *in vivo* : The acute toxicity tests were performed according to the semi-static method as described in the OECD guideline for testing of chemicals No. 203 "Test of acute toxicity for fish" (OECD, 1992). Based on the preliminary results, 10 individuals, randomly selected for every group, were exposed to a final concentration of 0 (solvent control), 25, 27.5, 30, 32.5, and 35 $\mu\text{g ml}^{-1}$ diethyl phthalate in seawater for 96 hrs. The seawater was maintained at a constant concentration of

Table 1 : Acute toxicity of flounder (*Paralichthys olivaceus*) exposed to different concentrations of diethyl phthalate (DEP) for 96 hrs

DEP concentration ($\mu\text{g ml}^{-1}$)	No. of fish exposed	Mortality (%)	LC ₅₀ value ($\mu\text{g ml}^{-1}$)
0	10	0	
25	10	0 $\pm$ 0	
27.5	10	50 $\pm$ 0	
30	10	50 $\pm$ 2.5	31.92 $\pm$ 0.16
32.5	10	75 $\pm$ 0.5	
35	10	100 $\pm$ 0

Fig. 4 : Light micrographs of transverse section of *Paralichthys olivaceus* kidney in 0 (control), 5, 10 and 15 $\mu\text{g ml}^{-1}$ diethyl phthalate exposure for 14 days (H&E staining). A: Control kidney tissues. Ut: Uriniferous tubules; E: Epithelium. B, C and D: Kidney tissues of flounder exposed to 5, 10 and 15 $\mu\text{g ml}^{-1}$ diethyl phthalate, respectively. En: Epithelium necrosis; He: Hemolytic erythrocytes. Scale bars: 25 μm

Fig. 5 : Effects of varied concentrations of diethyl phthalate on enzyme activities (ALT and LDH) in liver of *Paralichthys olivaceus*. Values given as mean $\pm$ S.E. * denotes significant difference from control ($P < 0.05$)

Fig. 6 : Effects of varied concentrations of diethyl phthalate on enzyme activities (ALT and LDH) in kidney of *Paralichthys olivaceus*. Values given as mean $\pm$ S.E. * denotes significant difference from control ($P < 0.05$)

diethyl phthalate by changing it at every 24 hr. Dead fishes were counted and removed immediately every day. The experiments were repeated three times. The 96 hr-LC₅₀ value was determined using Trimmed Spearman-Kärber estimation of LC₅₀. The 95% confidence limit value was calculated using the Probit method as described by Xiong (2000).

Histopathological examination in flounders : Based on the 96 hr-LC₅₀ value of diethyl phthalate obtained previously, three sublethal concentrations 5, 10 and 15 µg ml⁻¹ diethyl phthalate were chosen and used for the fish histological examination. Control fish were processed similarly. At the end of 14 days of diethyl phthalate exposure, the fishes from each test group, as well as the control group, were decapitated, and the liver and kidney were dissected out and fixed in Bouin's fixative for 48 hrs. They were then washed with running tap water overnight. After

dehydration in graded alcohol series, they were embedded in paraffin and sectioned at 6 µm. The sections were double stained with hematoxylin and eosin (H&E), mounted in neutral balsam for observation using an Olympus BX51 (Japan) microscope.

Biochemical parameters analysis in flounders : For biochemical analysis, the control and diethyl phthalate treated fishes were killed by decapitation and livers and kidneys were dissected out and homogenized in cold 0.85% saline using a glass homogenizer on ice. The final concentrations of the homogenates were adjusted to 10% (w/v). The homogenates were centrifuged at 12,000 g for 15 mins at 4 °C and used for the assays of enzyme activities. The biochemical parameters [alanine aminotransferase (ALT) and lactate dehydrogenase (LDH)] were determined by an AEROSSET automatic biochemical analyzer (Abbott, USA). All experiments were performed in triplicate.

All data of enzyme activities were evaluated by one-way ANOVA (SPSS V. 13.0 for Windows, tests: least significant difference, Tukey's honestly significant difference). Values were expressed as mean $\pm$ S.E., significance at $p < 0.05$.

Results and Discussion

Acute toxic effects of diethyl phthalate (100 to 800 $\mu\text{g ml}^{-1}$ for 24 hrs) were evaluated by NR uptake and MTT assays. As shown in Fig. 1, no significant cytotoxicity was noted below 100 $\mu\text{g ml}^{-1}$ diethyl phthalate. Instead, cell viability was excited at low dose. Afterwards, with diethyl phthalate concentrations increasing from 200 $\mu\text{g ml}^{-1}$ to 800 $\mu\text{g ml}^{-1}$, the cytotoxicity was obviously enhanced and showed a concentration-diethyl phthalate endent reduction. By linear regression analysis, the 24 hr- IC_{50} (95% confidence limits) diethyl phthalate values were 536.76 $\mu\text{g ml}^{-1}$ and 573.84 $\mu\text{g ml}^{-1}$ for NR and MTT, respectively. The cytotoxicity of diethyl phthalate to FG cells was similar in NR and MTT assays, regardless of the toxic endpoints employed.

Control FG cells are normally epithelioid in morphology as evidence of spindle shapes and attached firmly to the substratum with random orientations (Fig. 2 A). When FG cells were exposed to 100 $\mu\text{g ml}^{-1}$ diethyl phthalate, the cells showed normal morphology and their density was promoted (Fig. 2 B). When treated with 200 $\mu\text{g ml}^{-1}$ diethyl phthalate, the cellular morphology had a little adverse change, as seen by a trend of round shapes compared with the control group (Fig. 2 C). At 400 $\mu\text{g ml}^{-1}$ diethyl phthalate, the changes of cell morphology were much more obvious even some cells were detached from the cultural substratum (Fig. 2 D). Further, with 600 and 800 $\mu\text{g ml}^{-1}$ diethyl phthalate treatments for 24 hrs, an increasing number of FG cells became swollen, detached from the culture, presented a large number of lipid particles in cytoplasm, and eventually died (Fig. 2 E and F). Similar to NR and MTT assays, the morphological change trend of FG cells was also dependent on diethyl phthalate concentrations.

Previous studies have shown that diethyl phthalate has dual effects *i.e.*, low concentration increases cell proliferation, whereas high concentration lead to cell damage and inhibition (Kumar *et al.*, 2014, 2015). Here, the pattern of FG cells with diethyl phthalate exposure has shown similar response as above. It firstly caused cell proliferations at lower concentrations, which is agreed well with the reports about diethyl phthalate's effect on proliferation of MCF-7 and CHO cells (e.g. less than 100 μM) as an estrogenic compound (Hong *et al.*, 2005; Kumar *et al.*, 2014, 2015). With the increase of diethyl phthalate concentrations, the viability of FG cells was inhibited, and cellular morphologies were also adversely altered for 24hrs. These suggested that diethyl phthalate was acutely toxic to FG cells, and FG cell line may be considered to be a primary indicator of cytotoxicity as a bioassay model *in vitro*. As for diethyl phthalate toxicity mechanism in cell levels, it was concluded the impairments of lysosome and

mitochondria in terms of NR uptake and MTT assays. Its physiological defense mechanisms need further investigation in whole fish level, which was performed as follows.

The acute toxicity of diethyl phthalate in flounder fish was evaluated by 96 hr- LC_{50} in semi-static condition. The behavioral abnormalities of fish exposed to diethyl phthalate (25, 27.5, 30, 32.5, and 35 $\mu\text{g ml}^{-1}$) showed a concentration-dependent pattern in the 96 hr of exposure. When the diethyl phthalate concentration was less than 25 $\mu\text{g ml}^{-1}$, there were no mortality, but sluggishness and lethargic behavior could be observed. When its concentration was increased to 27.5 $\mu\text{g ml}^{-1}$, the exposed fish began to die, and the mortality rate was up to 50%. Finally, when diethyl phthalate concentration was 35 $\mu\text{g ml}^{-1}$, all exposed fish died in 96 hr of exposure. From the present investigation, 96 hr- LC_{50} for flounders was 31.92 $\mu\text{g ml}^{-1}$ (Table 1).

The lethal doses of diethyl phthalate was relatively high ($>1 \text{ g kg}^{-1}$ in oral or peritoneal administration) for guinea pigs, rats and mice (Api, 2001), however it was moderately toxic to fish (Staples *et al.*, 1997), 48 $\mu\text{g ml}^{-1}$ (96 hr- LC_{50}) to *Cyprinus carpio* (Barse *et al.*, 2007) and 50 $\mu\text{g ml}^{-1}$ (72 hr- LC_{50}) to *Cirrhinus mrigala* (Ghorpade *et al.*, 2002). As for *Paralichthys olivaceus*, the present acute toxicity result (96 hr- LC_{50} : 31.92 $\mu\text{g ml}^{-1}$) was similar to the above reported results on aquatic species. This information will be helpful in setting up exposure doses for studying of sublethal responses, including histological lesions and biochemical defense mechanisms.

The LC_{50} is considered as an important reference value for ecotoxicological studies in non-model and model animals for determining exposure doses of pollutants in risk assessment (Won *et al.*, 2016). On the other hand, the IC_{50} value of cells is also a promising estimation data in pollution monitoring due to its rapid and simple bioassay. However, here, the IC_{50} value was higher about one order of magnitude compared to the 96 hr- LC_{50} , meaning that *in vitro* cultured fish cells are more tolerant to DEP than living fish. Although the result testified the previous reports that *in vitro* data are not consistent with *in vivo* data (Gülden and Seibert, 2005; Yin *et al.*, 2007), the reasons for the higher tolerance of cultured fish cell line than living fish is not clear. One of the main reasons may be that cell lines and living animals belong to different bio-systems, and their action mode are different for *in vitro* cultured fish cells and living fish.

The structure of normal liver of fish showed polygonal shaped hepatocytes with a densely stained nucleus and congested blood vessels (Fig. 3 A). When the fish were treated with diethyl phthalate 5 $\mu\text{g ml}^{-1}$, the liver showed apparent vacuolation in hepatocyte cytoplasm (Fig. 3 B). For 10 $\mu\text{g ml}^{-1}$ diethyl phthalate treated fish, the structure of livers showed additionally a marked dilation of sinusoid space besides the hepatocyte vacuolation mentioned above (Fig. 3 C). When diethyl phthalate concentration increased to 15 μg

ml⁻¹, severe damages of liver architecture were observed, as evidence of nucleus pyknosis, hepatic sinusoid disorder accompanied with cloudy swelling, and degradation of cell membrane (Fig. 3 D). The effects were similar to other xenobiotics exposed fishes (*Channa punctata* Bloch) (Tabassum et al., 2016).

It is a quite well-known fact that drug toxicity is rarely limited only to one particular organ. As for the flounder kidneys, the control section showed normal structures of uriniferous tubules, in which tubular epithelial cells were ranged in order with clear nuclei (Fig. 4 A). When fish was treated with 5 µg ml⁻¹ diethyl phthalate, it was seen that epithelium boundary was obscured and the nuclei were also unapparent in uriniferous tubules, which indicated the beginning of a necrosis step (Fig. 4 B). When fish were exposed to higher concentrations (10 µg ml⁻¹ and 15 µg ml⁻¹), a more severe deterioration trend was observed including degradation of tubular epithelial cells and multiple hemorrhages in kidneys (Fig. 4 C and D). The renal histoarchitecture suggested that diethyl phthalate caused renal cellular damage and necrosis.

It was reported that significant changes in histological structure are associated with alterations in biochemical parameters (Haimanti et al., 2008; Husak et al., 2014). To investigate if these histopathological alterations, can cause dysfunction of these tissues of flounders in the study, biochemical parameters were evaluated at same diethyl phthalate-exposed concentrations.

As shown in Fig. 5 the activities of ALT and LDH increased significantly in the liver of flounders exposed to all diethyl phthalate concentrations ($p < 0.05$). The ALT levels 75%, 100% and 125% increase in the treatment groups (5, 10 and 15 µg ml⁻¹), respectively (Fig. 5A). ALT is clinically an important metabolic transaminase. Increased ALT levels indicated that diethyl phthalate stimulated transaminase activity due to the toxic injury caused by diethyl phthalate, which enhance tissue repair through protein turnover and amino acid metabolism. (Ghorpade et al., 2002; Jeon et al., 2013). LDH levels also showed a dramatic rise of 60%, 66% and 197% increase in 5, 10 and 15 µg ml⁻¹ diethyl phthalate concentration, respectively (Fig. 5B). The elevated activity of LDH in response to diethyl phthalate indicated an increase in anaerobic respiratory activity and production of more lactate for completion of metabolic process (Tripathi and Singh, 2013). In a word, the elevated levels of ALT and LDH demonstrated the metabolism and detoxification capacity was enhanced in liver tissue because of exposure of diethyl phthalate.

ALT and LDH enzyme activities were enhanced to various extents, especially at 10 and 15 µg ml⁻¹ DEP, showing a significant rise (ALT: 225% and 300%; LDH: 466% and 500%,) ($p < 0.05$). Kidney is reported as a major route for the excretion of xenobiotics. Fish kidney is a primary organ to be affected by toxicants in the aquatic environment (Thophon et al., 2003;

Qureshi et al., 2016). In the study, the up-regulation of enzyme activities (ALT and LDH) indicated metabolic change and damage response in kidney tissue. Hence, intoxication with DEP may potentially induce histological changes and modify the biochemical parameters in fish kidney. The pathological examinations and biochemical analysis in kidneys might be expected to be good indication of the study of DEP action mechanisms.

In conclusion, the study demonstrated that diethyl phthalate is a toxic substance both for fish and its cell line *in vitro* as well as *in vivo*. The cultured FG cells are applicable for a rapid estimation for diethyl phthalate acute toxicity. The experiment of *in vivo* toxicity of diethyl phthalate is a robust analytical method for studying diethyl phthalate toxic mechanism.

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