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Research Article

DI-ISONONYL PHTHALATE (DINP) AND DI-(2-ETHYLHEXYL)-PHTHALATE (DEHP) DISRUPTS ENDOCRINE FUNCTIONS IN THE FRESHWATER FISH, *OREOCHROMIS MOSSAMBICUS* (PETERS, 1852)

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ABSTRACT

Endocrine disrupting effects of two phthalate plasticizers namely di-isononyl-phthalate (DINP) and di-(2-ethylhexyl)-phthalate (DEHP) were evaluated in the freshwater fish, Oreochromis mossambicus. Male and female fishes were grouped separately and exposed to sublethal concentration of DINP (300 ppm) and DEHP (60 ppm) for 4, 7, 14, 30 and 60 days. At the end of every exposure period, blood serum was collected to determine the levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulating hormone (TSH), cortisol, testosterone and estradiol, and was compared with the respective control groups. The main findings of the study indicated that the levels of FSH and LH initially increased significantly ($P<0.05$) after the phthalates exposure, and decreased after 7 days onwards in both male and female fishes when compared to the corresponding control groups. DINP and DEHP treatment caused significant ($P<0.05$) decrease in the levels of TSH, cortisol and testosterone in time-dependent manner whereas the level of estradiol showed significant ($P<0.05$) reduction after 14 days of exposure in female fish without any significant changes in the male fish. The results suggest that exposure to phthalate plasticizers disrupts the hypothalamo-pituitary endocrine functions and this could be related to abnormal reproductive functions in the fish.

Keywords: *DINP, DEHP, FSH, LH, TSH, Estradiol, Testosterone.*

1. INTRODUCTION

Hypothalamo-pituitary-gonadal (HPG) axis is the highly conserved region in jawed vertebrates that regulates the synthesis and release of pituitary gonadotropin hormones namely follicle stimulating hormone (FSH) and luteinizing hormone (LH), which in turn control the gonadal growth and development [1]. In addition, endocrine feedback of gonad-derived activin and inhibin on the regulation of gonadotropins contributes to the synchronization of the HPG axis [2]. Most of the male and female reproductive disorders observed in fish population are associated to the exposure with chemicals possessing endocrine disrupting properties. The adverse impacts of endocrine disrupting chemicals (EDCs) in fish are identified by the assessment of several reproductive endpoints such as reproductive behaviour, delay in sexual maturity, gonado-somatic indices, level of vitellogenin, sperm parameters, activities of steroidogenic enzymes and changes in hormonal parameters [3].

Phthalates are the ubiquitous plasticizers widely used in broad variety of products including toys, personal care products, food packaging and medical devices, so as to provide flexibility and durability to the products. Phthalates are known as endocrine disruptors as they alter the levels of hormones in both terrestrial and aquatic vertebrates, either through the inhibition of hypothalamo-pituitary-gonadal axis or by the inhibition of gonadal gene expression thereby causing adverse health outcomes, but the mechanism remains unclear [4]. The widespread exposure and the possible teratogenic, carcinogenic, and endocrine disrupting actions focused the researchers to study the effects of phthalate plasticizers on aquatic organisms.

Fish are the only sensitive aquatic organism, and therefore used as suitable animal model to predict the impact of endocrine disrupting chemicals. On exposure of EDCs in aquatic ecosystems are known to cause several reproductive malfunctions in fish, such as reduced gamete production, decreased fecundity and spawning rate, inability of fertilization, alteration in the levels of hormones and so on. The entry of EDCs into the organisms is affected by series of factors including species, age, and sex, feeding behaviour, bioaccumulation, biomagnifications and biotransformation potential. Besides, several biotic and abiotic factors can influence the transfer of EDCs at trophic level within the aquatic population. The direct effects of EDCs on endocrine system provide significant implication on individual-level effects and facilitate to study the reproductive output of that population.

The endocrine system of fishes is found more similar to other higher vertebrates, composed of various glands located throughout the body. Gonadotropins include both follicle-stimulating hormone and luteinizing hormone that regulate gametogenesis and steroidogenesis, respectively, in the gonads, and promote the release of gametes from mature gonads [5]. The release of thyroid stimulating hormone from pituitary is influenced by certain other factors like temperature, salinity and photoperiod which help to adapt the fish against the changes in temperature and osmotic stress. Cortisol is the major corticosteroid hormone in teleost fish and chiefly modulates the stress generated by the exposure to environmental pollutants. Estradiol and testosterone are the major sex steroid hormones that display differential sex-specific and tissue-specific expression patterns that co-ordinates several reproductive events in fish [6, 7].

Toxicological studies provide broader impact on the effects of EDCs at individual and ecological level. In the present study, the endocrine disrupting effects of two phthalate plasticizers namely di-isononyl-phthalate (DINP) and di-(2-ethylhexyl)-phthalate (DEHP) were assessed in the freshwater fish, *Oreochromis mossambicus* by analyzing the changes in hypothalamo-pituitary gonadal hormones. The study provides basic information on the endocrine effects of phthalates in fish population thereby ultimately helps to understand the risk assessment of toxicants at population, community and ecosystem level.

2. MATERIALS AND METHODS

2.1. Test animal

Freshwater fish, *Oreochromis mossambicus* weighing 3.5 ± 0.75 g and length 5.5 ± 1.5 cm were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala, India. Fishes were transported to the laboratory with least disturbance and were acclimatized to the laboratory conditions prior to experiments with constant supply of water and good lighting (12 h light: 12 h dark) system in well-aerated and dechlorinated tubs (40 L capacity). The physico-chemical features of the tap water were estimated as per APHA guidelines [8] so that water temperature ranged between 28 ± 2°C, oxygen saturation of water was 70 and 100 %, and pH ranged as 6.5 to 7.5 throughout the experimental durations.

2.2. Chemicals

Di-isononyl phthalate (DINP; CAS No. 28553-12-0) of 99% purity and di-(2-ethylhexyl)-phthalate (DEHP; CAS No. 117-81-7) of 99.7% purity were obtained from Sigma Aldrich Chemical Co., USA. Commercial enzyme-linked immunosorbent assay (ELISA) kits for FSH, LH, TSH, cortisol, estradiol and testosterone were obtained from Diagnostic System Laboratories, Inc. Webster, Texas, USA.

2.3. Treatment

In group I, DINP at 300 ppm concentration dissolved in propylene glycol (1M) was exposed to fish for 4, 7, 14, 30 and 60 days. In group II, DEHP at 60 ppm concentration dissolved in propylene glycol (1M) was exposed to fish for 4, 7, 14, 30 and 60 days. Propylene glycol exposed group was maintained as vehicle control, and the group of fish without phthalates or propylene glycol was maintained as a negative control. Ten fishes were retained in each treatment groups, and the health status of the animal was continuously monitored throughout the experiment.

2.4. Sample preparation and hormone analysis

After every exposure period, fish were captured gently using small dip net and blood samples were collected from both male and female fishes by cardiac puncture method with the help of glass syringe into clean microcentrifuge tubes. Blood was centrifuged at 1700*g* for 15 min at 4°C to obtain serum for the analysis of hormones such as FSH, LH, TSH, cortisol, estradiol and testosterone, which were measured immediately using commercial ELISA kits. The assays were done strictly according to the procedure given along with the kits. For assaying serum hormones, all the samples and reagents were equilibrated with room temperature and were mixed thoroughly by gentle inversion prior to use. Standards, controls and unknowns were assayed in duplicate.

2.5. Statistical analysis

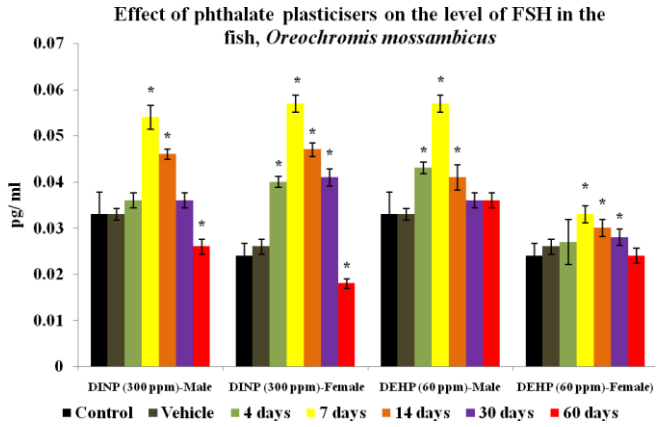
The results obtained in the experiment were analysed using statistical program SPSS 19.0 for Windows. The values of hormonal parameters were expressed as Mean ± SD for n = 10 animals/ group. Analysis of variance (ANOVA) was performed to determine significant differences of hormones among different groups. Differences in mean values were analyzed by Duncan's Multiple Range test and the probability level for all statistical tests was set significant at $p<0.05$ against the control groups.

3. RESULTS

3.1. Effects of DINP and DEHP on the level of FSH

Exposure of DINP at 300 ppm concentration showed slight increase in the level of FSH after 4 days, which significantly ($P<0.05$) increased after 7 days followed by time-dependent decrease though significant ($P<0.05$) reduction was found only after 60 days of treatment in male fish (Fig. 1). However, in female fishes, there was significant ($P<0.05$) increase in the level of FSH from 4 days of DINP exposure with significant ($P<0.05$) decrease after 60 days of treatment (Fig. 1).

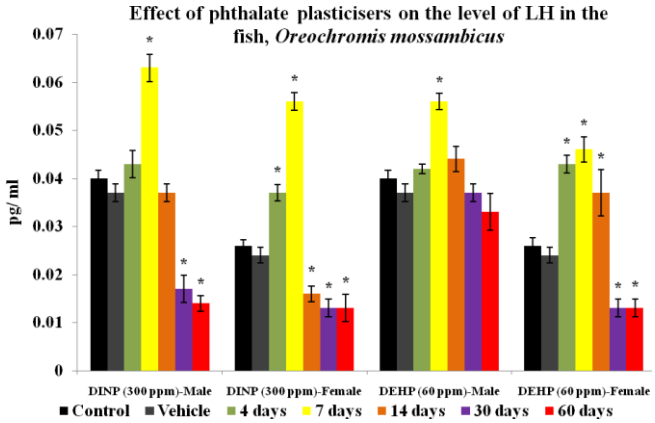
DEHP exposure at 60 ppm concentration in male fishes showed significant ($P<0.05$) increase after 4, 7 and 14 days and without significant changes after 30 and 60 days of treatment (Fig. 1). Female fishes on DEHP exposure showed significant ($P<0.05$) increase only in the groups of 7, 14 and 30 days when compared to the respective control groups (Fig. 1). In the present study, fishes exposed to propylene glycol (vehicle control) showed no significant hormonal changes in both male and female fishes when compared with solvent-free control group (Fig. 1).



3.2. Effects of DINP and DEHP on the level of LH

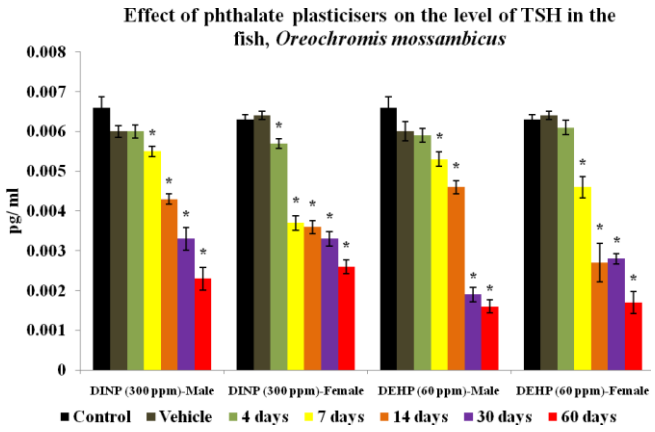
Male fish on exposure to DINP showed significant ($P<0.05$) increase in the level of LH only in 7 days treated group, with significant ($P<0.05$) reduction after 30 and 60 days (Fig. 2). DINP when exposed to female fishes showed significant ($P<0.05$) increase in the level of LH after 4 and 7 days with significant ($P<0.05$) and time-dependent decrease after 14 days of treatment onwards when compared to corresponding control groups (Fig. 2).

DEHP exposure in male fishes showed significant ($P<0.05$) increase in the level of LH only in 7 days exposed group without significant changes in other treatment groups (Fig. 2). However, in female fishes DEHP exposure caused significant ($P<0.05$) increase in the level of LH after 4, 7 and 14 days while significant ($P<0.05$) decrease was observed after 30 and 60 days of treatment (Fig. 2).



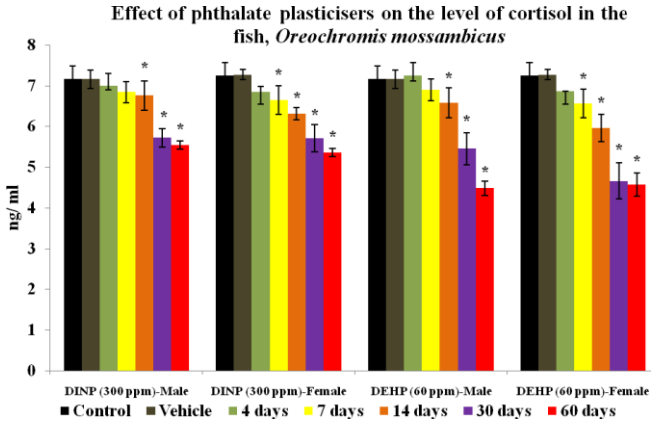
3.3. Effects of DINP and DEHP on the level of TSH

Exposure of DINP and DEHP showed significant ($P<0.05$) decrease in the level of TSH after 7 days in time-dependent manner without significant changes after 4 days in male fishes (Figure 3). Meanwhile, female fishes when exposed to DINP showed significant ($P<0.05$) decrease in the level of TSH in all treatment durations however, significant ($P<0.05$) and time-dependent reduction was observed only after 7 days of DEHP treatment when compared to corresponding control groups (Fig.3).



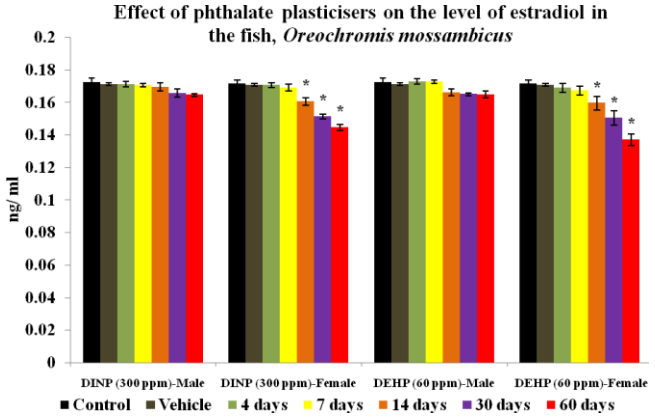
3.4. Effects of DINP and DEHP on the level of cortisol

A significant (P<0.05) and time-dependent decrease in the level of cortisol was observed in both male and female fishes after exposure to DINP and DEHP when compared to respective vehicle and negative control groups (Fig. 4).



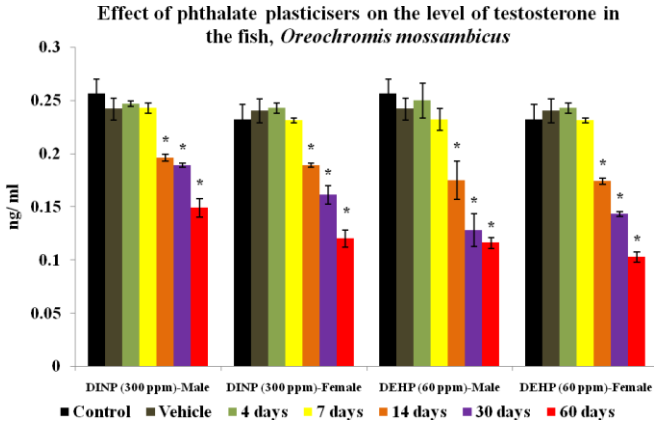
3.5. Effects of DINP and DEHP on the level of estradiol

DINP and DEHP exposure did not caused significant changes in the level of estradiol in male fishes, however, significant ($P<0.05$) reduction was observed in female fishes after 14 days of treatment in time-dependent manner (Fig. 5).



3.6. Effects of DINP and DEHP on the level of testosterone

The level of testosterone decreased significantly ($P<0.05$) after DINP and DEHP exposure in both male and female fishes when compared to the control groups (Fig. 6).



4. DISCUSSION

Aquatic ecosystems are highly vulnerable to the toxic effects of certain anthropogenic chemicals possessing endocrine disrupting properties. Phthalate plasticizers are well established environmental endocrine disruptors that pose potential reproductive health hazards by upsetting the normal functions of hypothalamo-pituitary gonadal (HPG) axis [9]. Phthalates are classified into two distinct groups, based on the number of carbon atoms in their alcohol chain, and also in relation to applications and toxicological properties. High phthalates or high molecular weight phthalates are with more than 7-13 carbons in their backbone thereby provides more permanency and durability whereas low phthalates or low molecular weight phthalates are with only 3-6 carbon atoms [9]. In the present study, di-isononyl phthalate (DINP) of high phthalates and di-(2-ethylhexyl)-phthalate (DEHP) of low phthalate group were chosen to evaluate the toxic effects on endocrine functions in the freshwater fish, *Oreochromis mossambicus*.

The purpose of the study was to simultaneously examine the endocrine disrupting effects of two selected phthalates, DINP and DEHP, on endocrine functions by the measurement of circulating hormone levels in the controlled laboratory condition. Exposure of DINP and DEHP showed an initial increase in the level of FSH followed by significant reduction at the end of 60 days of treatment in both male and female fishes. The results indicated that the initial up regulation of FSH could be due to the stimulatory effect of FSH on its own receptor mRNA as a result of estrogenic activity of phthalate plasticizers. However, prolonged exposure of DINP and DEHP could have stimulated the secretion of inhibin from gonads to down regulate FSH levels thereby disrupting the hypothalamo-pituitary functions. The results showed an agreement to another study when DEHP was exposed to adult female rats *in utero* showed an increase in the activities of gonadotropin releasing hormone that modulates FSH stimulation at HPG axis [10]. The mechanism underlying the positive association between phthalate exposure and serum FSH levels still remains unclear.

Similarly, exposure to DINP and DEHP initially increased the level of LH in both male and female fishes and on chronic exposure inhibited pituitary gland secretion by imbalance at HPG axis. Female marine medaka, *Oryzias melastigma*, has been shown to respond towards one of the phthalate plasticizers, DEHP at 0.1 mg/ L concentration by enhancing the gonadotropin

gene expression [11]. Thus phthalate exposure can interfere with the endogenous ligand of gondatropins and alter normal endocrine functions in the fish.

Thyroid stimulating hormone (TSH) has been involved in the processes of differentiation, growth, metabolism, and reproduction in fish regulated by hypothalamus-pituitary-thyroid (HPT) axis, which is responsible for the synthesis, secretion and metabolism of thyroid hormone [12]. The present results showed decrease in the level of TSH in male and female fishes after DINP and DEHP exposure thereby indicated that phthalates altered energy homeostasis and metabolic process in the exposed fish. Thyroid-disrupting effects of DEHP has been earlier documented by the decline in thyroid hormone levels through the activation of Ras/ Akt/ TRHr pathway thereby proving thyrotoxicity in clinical conditions [13]. The marked reduction of TSH expression in pituitary has also been associated to low testicular weight and reduction in the number of spermatozoa [14]. Cortisol is the principle corticosteroid in teleostian fishes regulated by corticotropin-releasing hormone that are released into circulation shortly after exposure to stress condition [15]. The present study observed a significant and time-dependent decrease in the level of cortisol in both male and female fishes after DINP and DEHP exposure thereby reflected the inhibitory effect of phthalates on hypothalamo-pituitary-adrenal axis. Similar observation has been reported when low level of DEHP was exposed to rats on alternate days for 14 days [16]. DINP exposure has been shown to decrease the activities of steroidogenic enzymes namely 3β- and 17β-hydroxysteroid dehydrogenase in ovary and testes of the fish *Oreochromis mossambicus* [17] and this could be correlated to the decline in the level of corticosteroid hormone.

DINP and DEHP exposure did not caused significant changes in the level of estradiol in male fishes, however, significant reduction was observed in female fishes thereby indicated that phthalates affects fish reproductive cycle that markedly reduce the growth of antral follicles, fecundity rate and ultimately leads to infertility [18]. The reduction in the levels of FSH and LH after chronic exposure of DINP and DEHP could have directly affected the ability of ovary to produce estradiol in normal level. The present findings illustrated that phthalates disrupted estrogen receptor function by mimicking the natural ligand and acts as agonist thereby declined the circulating level of estradiol.

Like many EDCs, DINP and DEHP decreased the level of testosterone in both male and female fishes thereby suggested to interfere with normal steroidogenesis by suppressing the expression of steroidogenic enzymes. DEHP has been shown to alter the functions of Sertoli and Leydig cells during development and inhibit testosterone production [19]. Anti-androgenic properties of DEHP have been demonstrated in human testis by the direct inhibition of testosterone synthesis in Leydig cells as a result of cytochrome CYP17 dysfunction [20]. Phthalate plasticizer such as di-(n-butyl)-phthalate has been shown to decrease the level of testosterone in fetal rat testis by the disruption in the patterns of gene expression that regulate cholesterol and lipid homeostasis or insulin signalling [21]. Thus the results suggested that phthalate exposure altered the normal circulating levels of hormones in both sexes of the fish which can be associated with endocrine dysfunction.

5. CONCLUSION

The influence of DINP and DEHP on the functions of hypothalamo-pituitary-gonadal axis was assessed by measuring the circulating level of serum hormones. The present findings demonstrated the disruption in the endocrine functions of the freshwater fish, *Oreochromis mossambicus*. The imbalance in hypothalamo-pituitary endocrine functions could disrupt reproductive ability of fish. Thus exposure of such phthalate plasticizers could affect the fish population in natural aquatic environment.

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