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Metabolic Stress Responses in *Labeo rohita* Subjected to Di (2-ethylhexyl) Phthalate (DEHP), Dibutyl Phthalate (DBP), and Diethyl Phthalate (DEP)

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

This study investigates the stress response of glucose and cholesterol levels in *Labeo rohita* (Rohu) upon exposure to three widely used phthalates: Di(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), and Diethyl phthalate (DEP). Phthalates, recognized for their endocrine-disrupting properties, are prevalent environmental contaminants. Specimens of *Labeo rohita* were subjected to environmentally relevant concentrations of DEHP, DBP, and DEP over a controlled period. Blood

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samples were collected periodically to measure fluctuations in glucose and cholesterol levels, serving as biomarkers of metabolic stress. The results indicate significant alterations in both glucose and cholesterol concentrations, with each phthalate demonstrating a distinct impact on these metabolic parameters. DEHP and DBP elicited more pronounced disruptions compared to DEP, underscoring their higher toxicity. These findings highlight the differential metabolic responses of *Labeo rohita* to various phthalates and underscore the ecological risks associated with phthalate contamination. The study advocates for stricter regulatory measures to mitigate phthalate pollution and protect aquatic ecosystems. This research enhances our understanding of the biochemical pathways affected by phthalate exposure and lays the groundwork for future investigations into the mechanisms underlying phthalate-induced stress in fish.

Keywords: Labeo rohita; phthalates; endocrine disruption; metabolic stress; aquatic toxicity.

1. INTRODUCTION

Phthalates are a group of synthetic chemicals extensively used as plasticizers in a wide range of consumer products, including plastics, cosmetics, and personal care items. Their widespread use has resulted in pervasive environmental contamination, particularly in aquatic ecosystems [1,2]. Among the various phthalates, Di(2-ethylhexyl) phthalate (DEHP), Dibutyl phthalate (DBP), and Diethyl phthalate (DEP) are notably ubiquitous due to their extensive applications in industry and consumer products [3,4].

Phthalates are well-documented endocrine disruptors, capable of interfering with hormonal balance and causing adverse health effects in both humans and wildlife [5,6]. In aquatic environments, fish are particularly vulnerable to phthalate exposure, which can lead to a range of physiological and biochemical disturbances, especially serum biochemical parameters, enzyme parameters, etc. Previous studies have highlighted the impact of phthalates on various aspects of fish biology, including reproduction, growth, and metabolic processes [7,8].

Labeo rohita, commonly known as Rohu, is a freshwater fish of considerable ecological and economic importance in South Asia. As a model organism, *Labeo rohita* provides valuable insights into the environmental stress responses of fish [9]. Metabolic parameters such as glucose and cholesterol levels serve as candidate biomarkers for assessing stress responses in fish. Glucose is a primary energy source, and its levels can indicate alterations in metabolic and stress pathways [10]. Similarly, cholesterol is an essential component of cellular membranes and is involved in steroidogenesis; its levels can reflect disruptions in lipid metabolism and endocrine function [11].

This study aims to investigate the stress response of glucose and cholesterol levels in *Labeo rohita* upon exposure to three ubiquitous phthalates: DEHP, DBP, and DEP. By analyzing the variations in these metabolic parameters, we seek to elucidate the differential impacts of these phthalates on *Labeo rohita* and contribute to the understanding of phthalate-induced stress mechanism in fish.

2. MATERIALS AND METHODS

2.1 Experimental Design

Labeo rohita specimens were obtained from a local fish farm and acclimatized in laboratory conditions for two weeks in large, aerated tanks with a 12-hour light/12-hour dark cycle. The fish were fed a commercial diet and water parameters were maintained at optimal levels (temperature: 25 ± 2°C, pH: 7.2-7.5, dissolved oxygen: 6.5-7.5 mg/L). Prior to the experiment, fish were fasted for 24 hours to standardize metabolic conditions. The experimental fishes were exposed to the plasticizers by method of immersion and osmotic infiltration where the three (DEHP, DBP and DEP) selected Phthalate plasticizer compounds were mixed with acetone for complete dissolution as most of the phthalates were mixed with acetones to produce commercial personal care products and they are then added to the respective tanks containing fishes.

2.2 Assessment of Median Lethal Concentration (Lc_{50}) Value

The experimental dosage of Technical grade DEHP, DBP and DEP dissolved in Acetone at required concentration was added to the experimental fish tanks. Before the exposure period, the experimental fishes were made to starve for 24 hours. The experiment was carried

out to determine the dosage range for further examination. At 72-hour durations, the relative mortality for various experimental dosages was recorded. The LC_{50} value and its 95% confidence limits were determined by Behreus and Karbeur [12].

2.3 Design of Sub - Acute Toxicity Study

Based on the results of the LC_{50} doses of DEHP, DBP and DEP were selected for the regular sub-acute toxicity of 30 days. Doses which comprised of $1/25^{th}$ of LC_{50} values (0.3 mg/L,0.15 mg/L, 0.25 mg/L) for DEHP, DBP and DEP, respectively were selected, following the procedures of Poopal et al., [13] and Paget [14]. In the present investigation, *Labeo rohita* of uniform body weight and age group were used for assessing the effects of 30day aqueous exposure. The animals were categorized into five groups A, B, C, D and E with each group comprising of six animals as suggested by Muller and Kley [15]. The doses were selected based on Mackay and Elliott [16], Brown (1980).

The control animals in group A were untreated and group B animals were treated with acetone which is used as vehicle which served as positive control. Group C, D and E were treated with $1/25^{th}$ of LC_{50} values (0.012 mg/L, 0.006 mg/L, 0.01 mg/L) of DEHP, DBP and DEP respectively. Toxicants dosage level was maintained with care in order to calculate the fluctuations in body weight, behavioral changes and food consumption in experimental animals.

2.4 Effect of Sub – Acute Dosage of Dehp (0.012 Mg/L), Dbp (0.006 Mg/L) and Dep (0.010 Mg/L) on Body Weight Changes

In sub-acute toxicity the animals were grouped as A, B, C, D, and E. Group A served as control, Group B was treated with Acetone which served as positive control, Group C, D and E were treated with 0.012 mg L^{-1} of DEHP, 0.006 mg L^{-1} of DBP and 0.010 mg L^{-1} of DEP. The animals were weighed every week from the beginning of experiment to 30 days. The mean and sample standard deviation (n -1), of body weight at day 30 was calculated from the data obtained.

2.5 Collection of Blood for Serum Biochemical Parameters

Blood was drawn from fish using plastic disposable syringes, fitted with 26 gauge needle, which contained heparin to prevent clogging. The blood samples were centrifuged at 10,000 rpm for 15 min and the plasma was separated for serum biochemical assays.

2.6 Serum Biochemical Analysis

The blood glucose was estimated as per method of O - toluidine [17], Total serum cholesterol was estimated by King and Wootton [18].

2.7 Statistical Analysis

The results obtained in the experiments were analysed using statistical program SPSS 16.0 for Windows. The values were expressed as Mean ± SD for n = 6 animals/ group. One way Analysis of variance (ANOVA) was performed to determine significant differences in values among different treated groups with respect to control. Differences in mean values were analysed 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 Median Lethal Concentration (LC_{50}) of DEHP

The 72 hrs median lethal concentration (LC_{50}) of DEHP for *Labeo rohita* was recorded as 300 mg/L body weight. The LC_{50} value was calculated by constructing the regression line, taking test doses and their corresponding mortalities in logarithmic values using Behreus and Karbeur [12] (Fig. 1).

3.2 Median Lethal Concentration (LC_{50}) of DBP

The 72 hrs median lethal concentration (LC_{50}) of DBP for *Labeo rohita* was recorded as 150 mg/L body weight. The LC_{50} value was calculated by constructing the regression line, taking test doses and their corresponding mortalities in logarithmic values using Behreus and Karbeur [12] (Fig. 2).

3.3 Median Lethal Concentration (LC_{50}) of DEP

The median lethal concentration (LC_{50}) of DEP for *Labeo rohita* was recorded as 250 mg/L body weight in 72 hours. The LC_{50} value was calculated by constructing the regression line,

taking test doses and their corresponding mortalities in logarithmic values using Behreus and Karbeur [12] (Fig. 3).

4. EFFECT OF SUB - ACUTE DOSAGE OF DEHP (0.012 MG/L), DBP (0.006 MG/L) AND DEP (0.010 MG/L) ON THE BLOOD GLUCOSE AND SERUM CHOLESTROL OF *Labeo rohita*

4.1 Blood Glucose

In the sub – acute experimental groups C, D and E the glucose content in the blood of *Labeo rohita* was observed to be 9, 6, and 8 mg/dl respectively. On the other hand in Control group A and acetone treated group B the values were recorded to be 22 and 21 mg/dl respectively. When the test of significance was performed between the control and experimental groups at 5% probability level, a decrease in glucose content was observed in groups B, C, D and E respectively, (Table 1, Fig. 4).

4.2 Serum Cholesterol

When DEHP - 0.012 mg/L, DBP - 0.006 mg/L and DEP - 0.010 mg/L treated *Labeo rohita,* in groups C, D and E were subjected to estimation of cholesterol content, the analysis showed the values to be 19, 11 and 17 mg/dl respectively, parallel to this the values in the Control group A and Acetone treated group B was found to be 31 and 29 mg/dl respectively. Further when the cholesterol values of group A, B, C, D and E were subjected to test of significance at 5% probability level revealed a progressive decrease in cholesterol content of group B and a sharp reduction in cholesterol content of group C, D and E, (Table 1, Fig. 4).

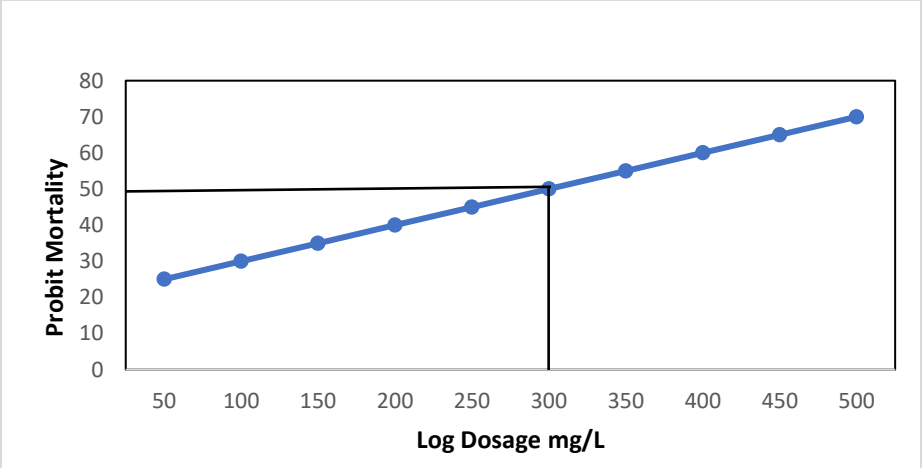

Fig. 1. Graph representing LC50 for DEHP

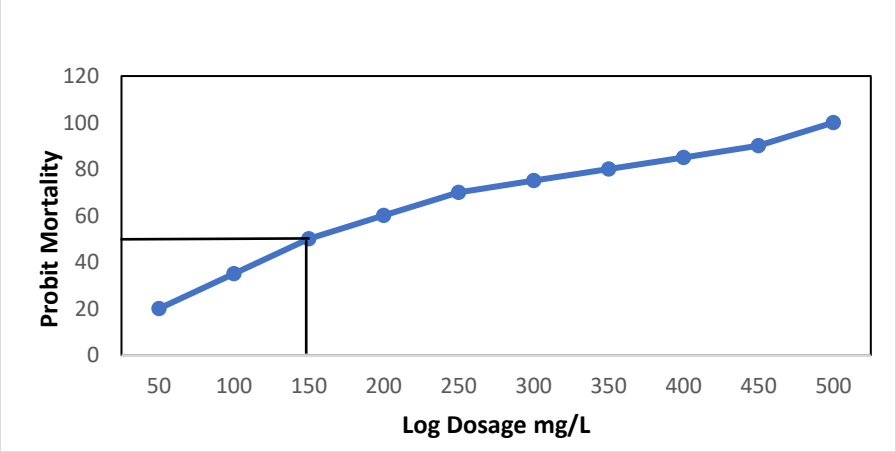

Fig. 2. Graph representing LC50 for DBP

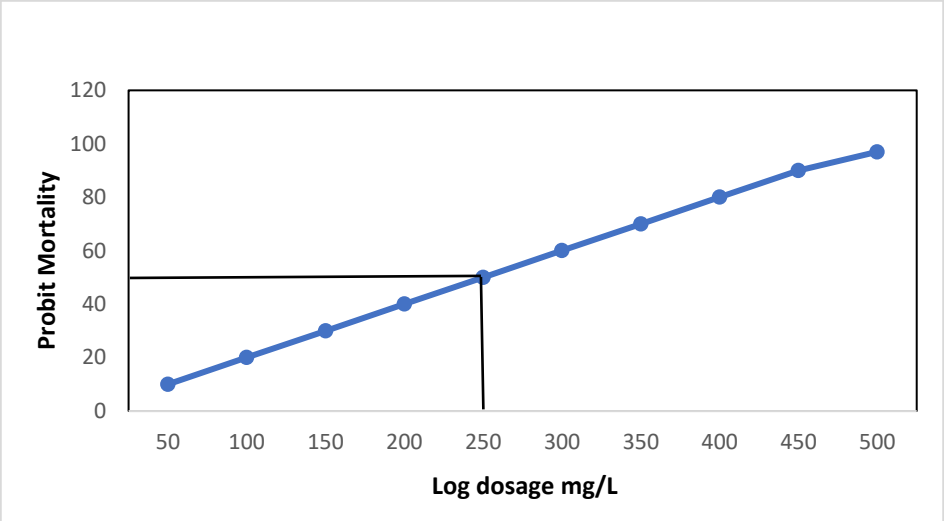

Fig. 3. Graph representing LC50 for DEP

Table 1. Effect of sub - acute dosage of dehp (0.012 mg/l), dbp (0.006 mg/l) and dep (0.010 mg/l) on blood glucose and serum cholestrol of *Labeo rohita*

Treatment	Blood Glucose (mg/dl)	Serum Cholesterol (mg/dl)
Group A - Control	22 ± 0.03 (n = 6)	31 ± 0.02 (n = 6)
Group B – Treated withAcetone	21 ± 0.05 (n = 6)	29 ± 0.01 (n = 6)
Group C – Treated withDEHP (0.012 mg/L)	9 ± 0.022 (n = 6)	19 ± 0.041 (n = 6)
Group D – Treated withDBP (0.006 mg/L)	6 ± 0.01 (n = 6)	11 ± 0.032 (n = 6)
Group E – Treated withDEP (0.010 mg/L)	8 ± 0.036 (n = 6)	17 ± 0.028 (n = 6)

Values are expressed as ± standard deviation
Values given in parenthesis are number of animals.
Treated groups are significantly different from control at 5% probability level.

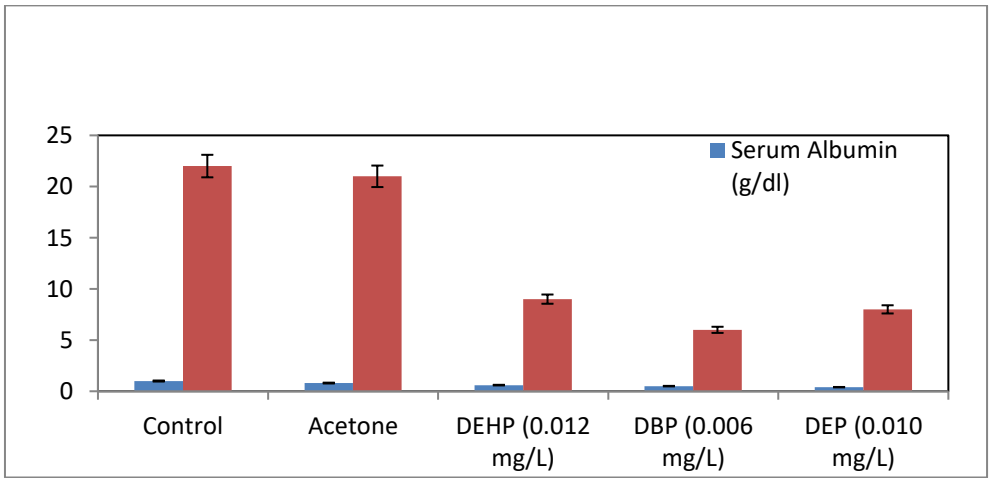

Fig. 4. Effect of sub - acute dosage of dehp (0.012 mg/l), dbp (0.006 mg/l) and dep (0.010 mg/l) on blood glucose and serum cholestrol of *Labeo rohita*

5. DISCUSSION

This study aimed to investigate the stress response of glucose and cholesterol levels in *Labeo rohita* upon exposure to three ubiquitous phthalates: DEHP, DBP, and DEP. The findings reveal significant alterations in both glucose and cholesterol concentrations, indicating a metabolic stress response induced by phthalate exposure.

5.1 Glucose Response

Our results showed that exposure to DEHP, DBP, and DEP led to a significant decrease in plasma glucose levels compared to the control group. This hypoglycemic response can be attributed to the activation of the hypothalamic-pituitary-interrenal (HPI) axis, which is analogous to the hypothalamic-pituitary-adrenal (HPA) axis in mammals [10]. The stress-induced release of cortisol, a primary stress hormone in fish, promotes gluconeogenesis and glycogenolysis, resulting in sharp decline in glucose levels [19].

The differential impact of the three phthalates on glucose levels suggests varying degrees of endocrine disruption. DEHP and DBP caused more pronounced increases in glucose levels compared to DEP, indicating their higher potency in eliciting stress responses. This is consistent with previous studies showing that DEHP and DBP have stronger endocrine-disrupting effects than DEP [3,5].

5.2 Cholesterol Response

Cholesterol levels in *Labeo rohita* also exhibited significant alterations upon exposure to the phthalates. Both DEHP and DBP exposure resulted in down regulation of cholesterol levels, whereas DEP had a comparatively lesser effect. Cholesterol is a critical component of cellular membranes and a precursor for steroid hormones. The observed hypocholesterolemia could be a compensatory mechanism to maintain cellular integrity and support increased steroidogenesis under stress conditions [11].

The decline in cholesterol levels can also be linked to disrupted lipid metabolism, a common effect of endocrine disruptors [6]. Phthalates are known to interfere with lipid homeostasis, leading to altered cholesterol synthesis and transport [20,21]. The greater impact of DEHP and DBP on cholesterol levels reinforces their higher endocrine-disrupting potential.

5.3 Ecological and Environmental Implications

The significant metabolic disruptions observed in *Labeo rohita* upon exposure to DEHP, DBP, and DEP underscore the ecological risks posed by these phthalates. Aquatic organisms, especially fish, are particularly vulnerable to endocrine disruptors due to their constant exposure to contaminated water. The metabolic stress responses observed in this study may have broader implications for fish health, including impaired growth, reproduction, and survival [7].

Given the widespread use of phthalates and their persistence in the environment, there is an urgent need for stringent regulatory measures to limit phthalate pollution. Effective wastewater treatment processes and the development of safer alternatives to phthalates are critical to mitigating their impact on aquatic ecosystems [2].

6. CONCLUSION AND FUTURE DIRECTIONS

This study highlights the significant metabolic stress responses in *Labeo rohita* upon exposure to DEHP, DBP, and DEP. The differential effects of these phthalates on glucose and cholesterol levels provide insights into their varying endocrine-disrupting potentials. Future research should focus on elucidating the underlying mechanisms of phthalate-induced metabolic disruptions and exploring the long-term impacts on fish health and population dynamics. Additionally, studies on the effectiveness of different mitigation strategies to reduce phthalate contamination in aquatic environments are warranted.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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