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GESTATIONAL CADMIUM AND DEHP CO-EXPOSURE ALTERS MATERNAL ENDOCRINE HOMEOSTASIS AND OFFSPRING HORMONAL PROGRAMMING

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ABSTRACT Human exposure to endocrine-disrupting chemicals typically occurs as complex mixtures rather than single compounds, yet toxicological assessments often focus on individual chemicals. To address this limitation, the present study examined the neuroendocrine and metabolic effects of gestational exposure to cadmium (Cd) and di-(2-ethylhexyl) phthalate (DEHP), administered individually and in combination using a phased experimental design. In Phase I, graded gestational exposures were used to establish dose–response relationships for region-specific oxidative stress biomarkers. Nonlinear regression analysis generated effective concentration (EC50) values, identifying biologically relevant sub-lethal doses (Cd: 1 mg/kg; DEHP: 100 mg/kg body weight). Phase II employed these EC50-defined doses to evaluate the cumulative effects of gestational exposure on maternal–offspring endocrine programming. Results revealed pronounced oxidative imbalance in offspring brain regions, with the hippocampus emerging as a primary target. Exposure to Cd or DEHP alone significantly elevated oxidative stress markers, while co-exposure produced a moderated yet significant response suggestive of an adaptive brain-sparing effect. Maternal exposure was associated with sustained activation of the hypothalamic–pituitary–adrenal axis, reflected by hypercortisolism and concurrent thyroid and metabolic disturbances. Offspring growth patterns paralleled maternal stress responses, indicating transgenerational endocrine disruption. Overall, gestational co-exposure to Cd and DEHP produced complex, non-additive neuroendocrine effects, highlighting the importance of EC50-guided dosing and nonlinear modeling in mixture toxicology and developmental risk assessment.

KEYWORDS : Cadmium; DEHP; Gestational Co-exposure; Endocrine Disruption; Mixture Toxicology; Developmental Programming

INTRODUCTION

In the modern industrial era, exposure to environmental pollutants, particularly endocrine-disrupting chemicals (EDCs), has become a significant global health concern. The World Health Organization and the United Nations Environment Programme have identified EDCs as substances of major concern due to their capacity to interfere with hormonal signaling even at very low concentrations[1]. While most toxicological studies focus on individual chemicals, real-world exposures occur as complex mixtures, emphasizing the need for mixture-based risk assessment approaches[2]. Among the most widespread contaminants are the plasticizer di-(2-ethylhexyl) phthalate (DEHP) and the heavy metal cadmium (Cd). DEHP is widely used in plastics and medical devices[3], whereas cadmium enters ecosystems through industrial emissions and agricultural runoff and subsequently accumulates in the food chain[4]. Consequently, simultaneous exposure to DEHP and cadmium is environmentally realistic[3,5], particularly during sensitive developmental periods such as gestation and lactation. According to the Developmental Origins of Health and Disease (DOHaD) hypothesis, environmental insults during critical developmental windows can permanently alter physiological systems and increase susceptibility to chronic diseases later in life[6]. Despite their chemical differences, DEHP and cadmium share several toxicological pathways[3,5]. DEHP disrupts steroidogenesis and the hypothalamic–pituitary–thyroid (HPT) axis[3], whereas cadmium acts as a metalloestrogen and neurotoxicant with high bioaccumulation and a long biological half-life[7]. Both toxicants induce oxidative stress by increasing reactive oxygen species and weakening antioxidant defenses such as superoxide dismutase (SOD) and catalase (CAT)[7], suggesting that combined exposure may produce additive or synergistic effects. Traditional toxicity metrics such as LD_{50} primarily reflect acute mortality and fail to capture subtle endocrine disturbances associated with chronic exposure[6,8]. In contrast, the effective concentration (EC_{50}) provides a more biologically relevant indicator of sub-lethal physiological disruption[3]. Therefore, the present study adopted a two-phase design: Phase I determined EC_{50} values of DEHP and cadmium using oxidative stress biomarkers (lipid peroxidation, SOD, and CAT), and Phase II evaluated the effects of gestational co-exposure on the maternal–fetal endocrine axis and neuroendocrine stress responses in weaning offspring[9,10]

MATERIALS AND METHODS

Experimental Animals and Ethics

Adult female Wistar rats procured from Sri Venkateswara Enterprises (Bangalore) were kept under standard laboratory conditions (22 ± 2 °C, 12-hour light-dark cycle) with free access to food and water. All experimental procedures followed the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and received approval from the Institutional Animal Ethics Committee (IAEC No: 402/GO/Re/01/CPCSEA/002).

Chemicals and Toxicity exposure- Cadmium chloride and di-(2-ethylhexyl) phthalate (DEHP) of analytical grade were procured from Sigma-Aldrich Ltd. DEHP was dissolved in olive oil as the vehicle control. Doses were selected based on preliminary EC50 screening using oxidative stress biomarkers to ensure biological relevance without overt toxicity. DEHP was administered at 100 mg/kg body weight (EC50-defined biologically effective doses eliciting oxidative response), while cadmium was given at 1 mg/kg body weight (EC50 window for oxidative markers). Combined exposure used concurrent administration of both at these doses to assess integrated toxicological effects under non-lethal conditions.

Experimental Design

Pregnant Wistar rats were randomly assigned to five groups (n = 6): control, vehicle control (olive oil), cadmium (1 mg/kg), DEHP (100 mg/kg), and combined DEHP + cadmium. Treatments were administered orally by gavage throughout gestation, with indirect pup exposure during lactation via maternal transfer. Maternal blood samples were collected on gestational day (GD)-19 following euthanasia for hormonal analysis. Offspring remained with dams until weaning and were euthanized on postnatal day (PND)-21 for biochemical and hormonal assessments.

Phase I: Determination of EC_{50} Values- Pregnant dams received graded doses of DEHP (100–500 mg/kg) and cadmium (1–4 mg/kg) from GD-1 through lactation to establish dose–response relationships. Oxidative stress in offspring cortex and hippocampus was evaluated by measuring lipid peroxidation (LPO) and antioxidant enzyme activities—superoxide dismutase (SOD) and catalase (CAT). EC_{50} values were estimated using nonlinear regression (four-parameter logistic model with Levenberg–Marquardt optimization) in GraphPad Prism 5.0.

Oxidative Stress Biomarkers- Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation through the thiobarbituric acid (TBA) reaction, quantified spectrophotometrically at 535 nm following the method of Niehaus and Samuelsson[11]. SOD activity was measured by the inhibition of epinephrine auto-oxidation at 480 nm according to Misra and Fridovich[12]. CAT activity was

determined by monitoring hydrogen peroxide decomposition at 240 nm using the method of Aebi[13].

Phase II: Exposure Study- Based on Phase I EC50 results, dams were exposed to cadmium (1 mg/kg) and DEHP (100 mg/kg), individually or in combination, from GD-1 to PND-21. These doses represented biologically relevant sub-lethal exposures producing measurable oxidative and endocrine responses. Maternal body weight and feed intake were recorded weekly. On PND-21, offspring brains were collected and the organosomatic index (OSI) was calculated as (organ weight/body weight) × 100. Maternal serum (GD-19) was analyzed for cortisol, estradiol, and thyroid hormones, while pup serum (PND-21) was assessed for cortisol and thyroid hormones.

Hormonal Analysis- Blood samples were collected by cardiac puncture, centrifuged (3000 rpm, 15 min), and serum isolated. Hormonal assays were performed using automated chemiluminescent immunoassay (CLIA). Thyroid hormones (fT3, fT4, TSH) were measured by non-competitive immunoassay, while estradiol and cortisol were quantified by competitive CLIA. Detection limits were 0.01 ng/dL (fT4), 0.02 pg/mL (fT3), 0.01 ng/mL (TSH), 1.6 pg/mL (estradiol), and 0.1 µg/dL (cortisol). Cortisol and estradiol analyses were conducted using a clinical chemistry autoanalyzer at Vetlesions Laboratory, Bangalore[14]

Statistical Analysis - Data were analyzed using SPSS 20.0 and expressed as mean ± SEM. As data were not normally distributed (Shapiro–Wilk test, $p < 0.05$), group differences were assessed using the Kruskal–Wallis test followed by Dunn's post hoc comparisons. Statistical significance was set at $p < 0.05$.

RESULTS

Phase I: Determination of Oxidative Stress EC_{50}

In this study, the dose–response analyses demonstrated a clear dose-dependent elevation in oxidative stress markers following cumulative exposure to both DEHP and cadmium (Figs. 1 and 2). In the DEHP-treated groups, progressive increase in lipid peroxidation was observed in both the cortex and hippocampus, with EC_{50} values of 103.7 mg/kg and 102.2 mg/kg, respectively. The antioxidant enzymes exhibited comparable dose–response behavior, with superoxide dismutase (SOD) and catalase (CAT) activities declining at similar threshold concentrations clustering near 100 mg/kg. In contrast, cadmium exhibited markedly higher intrinsic toxicity, as reflected by substantially lower EC_{50} values for lipid peroxidation, calculated at 1.009 mg/kg in the cortex and 1.097 mg/kg in the hippocampus. Based on these oxidative stress EC_{50} determinations, 100 mg/kg DEHP and 1 mg/kg cadmium were identified as biologically effective sub-lethal doses and were subsequently selected for the Phase II interaction study (Table 1).

Phase II: Maternal Systemic and Endocrine Toxicity

Maternal exposure to EC_{50}-defined doses of DEHP and cadmium induced pronounced systemic toxicity, particularly under co-exposure conditions. As illustrated in Figure 3, dams in the combined exposure group exhibited significant growth suppression by gestational day (GD)-21, characterized by a 14.1% reduction in body weight and a concurrent 7.1% decline in feed intake. The observed systemic stress coincided with marked endocrine disruption (Table 2; Fig.5). This systemic burden was most profoundly reflected in the adrenal response; the co-exposure group showed a staggering 968.8% surge in serum cortisol, signaling extreme physiological stress and HPA-axis activation. This stress coincided with overt primary hypothyroidism across all treated groups. In the co-exposure group, thyroid-stimulating hormone (TSH) increased by 141.7%, while circulating free T_3 and T_4 levels were substantially depleted (-66.7% and -52.0%, respectively). Furthermore, serum estrogen concentrations were significantly suppressed, with the combined exposure producing the most severe decline (-31.3%). Together, these data indicate that the DEHP+Cd mixture exacerbates ovarian and thyroid endocrine toxicity while inducing a massive systemic stress response.

Phase II: Offspring Developmental Toxicity

In this study, offspring growth patterns broadly reflected maternal systemic stress. As shown in Figure-4, pups from the co-exposure group exhibited severe intrauterine growth restriction, with birth weights reduced by 27.6% compared to controls. Although partial compensatory growth was observed by weaning (PND 21), body weights remained significantly compromised. Despite overall somatic growth restriction, brain development was preferentially preserved across exposure groups. This was reflected by a significant elevation in the organosomatic index (OSI) in treated offspring (Fig. 4). Cadmium- and DEHP-exposed pups exhibited marked increases in OSI (+43.9% and +39%, respectively), while the co-exposure group showed a moderated but still significant elevation (+5.3%), collectively indicating a clear brain-sparing pattern.

Phase II: Offspring Endocrine Dysregulation

The offspring endocrine profiles revealed profound hormonal dysregulation distinct from maternal responses (Table 3; Fig. 5). In this study, both individual and their co-exposures led to pronounced activation of the hypothalamic–pituitary–adrenal (HPA) axis, as indicated by marked hypercortisolism. The cortisol levels increased by 250% in the DEHP group and remained critically elevated in co-exposure group (+216.7%). In contrast to the hypothyroid state observed in dams, the co-exposed offspring displayed a paradoxical thyroid profile characterized by significant suppression of TSH (−68.4%) alongside a concomitant elevation in free thyroxine (FT_4; +30.2%).

Table 1: Calculated EC50 Values (mg/kg/bw) for Oxidative Stress Markers

Biomarkers	Tissues	DEHP EC50 (mg/kg)	Cadmium EC50 (mg/kg)
Lipid Peroxidation (LPO)	Cortex	103.7	1.009
	Hippocampus	102.2	1.097
Superoxide Dismutase (SOD)	Cortex	97.7	0.940
	Hippocampus	91.2	1.026
Catalase (CAT)	Cortex	112.2	0.978
	Hippocampus	110.9	1.069

Note: EC_{50} values represent the effective concentration required to induce 50% of the maximal oxidative response based on nonlinear regression analysis.

Table 2: Effects of Perinatal Exposure on Maternal Physiology and Endocrine Profile at Late Gestation (GD 19-21)

Parameters		Control	Cadmium (Cd)	Vehicle Control	DEHP	DEHP + Cd
Metabolic Indices (GD 21)	Body Weight (g)	311.3 ± 0.49	265.2 ± 1.62ns (-14.8%)	315.5 ± 0.22	267.0 ± 1.80** (-15.4%)	271.0 ± 0.37* (-14.1%)
	Feed Intake (g/day)	20.5 ± 0.04	19.4 ± 0.05ns (-5.4%)	19.8 ± 0.03	17.7 ± 0.08** (-10.6%)	18.4 ± 0.06* (-7.1%)
	Fluid Intake (g/day)	24.9 ± 0.04	23.8 ± 0.03* (-4.4)	23.3 ± 0.04	21.9 ± 0.04* (-6.0)	21.8 ± 0.03** (-6.4)
Endocrine profile (GD 19)	Cortisol (µg/dL)	0.3 ± 0.03	2.5 ± 0.05* (+733. 3%)	0.32 ± 0.04	2.7 ± 0.05* (+743.8)	3.42 ± 0.08** (+968.8 %)
	TSH (ng/dL)	1.3 ± 0.02	2.3 ± 0.07ns (+76.9%)	1.2 ± 0.03	2.7 ± 0.06ns (+125.5)	2.9 ± 0.02*** (+141.7 %)
	Free T3 (pg/dL)	0.5 ± 0.03	0.3 ± 0.02ns (-40.0%)	0.6 ± 0.02	0.3 ± 0.02** (-50.0%)	0.26 ± 0.02*** (-66.7%)
	Free T4 (ng/dL)	2.4 ± 0.04	1.6 ± 0.11ns (-33.3%)	2.5 ± 0.06	1.3 ± 0.06** (-48.0%)	1.2 ± 0.06*** (-52.0%)
	Estrogen (ng/dL)	5.43 ± 0.61	3.88 ± 0.41** (-28.5%)	5.24 ± 0.51	3.95 ± 0.73ns (-24.6%)	3.6 ± 0.44** (-31.3%)

Values are expressed as Mean ± SEM (n=6). Percentage change relative to control is indicated in parentheses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Control.

Table 3: Developmental and Endocrine Outcomes in Offspring at Weaning (PND 21)

Parameters		Control	Cadmium (Cd)	Vehicle Control	DEHP	DEHP + Cd
Metabolic Indices	Final Body Weight (g)	33.7 ± 0.25	21.8 ± 0.31** (-35.3%)	34.3 ± 0.09	22.8 ± 0.1** (-33.5%)	28.4 ± 0.15* (-17.0%)

	Brain OSI (%)	4.1 ± 0.14	5.9 ± 0.29* (+43.9%)	4.1 ± 0.10	5.7 ± 0.23* (+39. 0%)	4.2 ± 0.10* (+5.3%)
Endocrine profile (GD 22)	Cortisol (µg/dL)	0.5 ± 0.01	1.3 ± 0.06ns (+160. 0%)	0.6 ± 0.06	2.1 ± 0.05** (+250. 0%)	1.9 ± 0.06ns (+216. 7%)
	TSH (ng/dL)	1.7 ± 0.06	3.7 ± 0.05ns (+117. 6%)	1.9 ± 0.06	0.7 ± 0.06ns (-63.2%)	0.6 ± 0.06ns (-68.4%)
	Free T3 (pg/mL)	3.2 ± 0.2	2.3 ± 0.2ns (-28.1%)	2.5 ± 0.2	5.5 ± 0.4ns (+120. 0%)	0.9 ± 0.2** (-64.0%)
	Free T4 (ng/dL)	4.6 ± 0.03	3.7 ± 0.03ns (-19.6%)	4.3 ± 0.04	5.2 ± 0.05ns (+20. 9%)	5.6 ± 0.07* (+30. 2%)

Values are expressed as Mean ± SEM (n=6). Percentage change relative to control is indicated in parentheses. * $p < 0.05$, ** $p < 0.01$ vs Control.

DISCUSSION

This study evaluated the combined toxic effects of perinatal exposure to di-(2-ethylhexyl) phthalate (DEHP) and cadmium (Cd), adopting a mixture-toxicity framework rather than the conventional single-chemical approach, consistent with recommendations by Yan et al.[2]. EC_{50} values were derived using nonlinear regression (Levenberg–Marquardt optimization), enabling robust estimation of dose–response relationships for oxidative stress biomarkers—lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT)—in the cortex and hippocampus (Table 1). Cadmium exhibited markedly higher potency, with EC_{50} values near 1 mg/kg across markers and brain regions, compared with 91–112 mg/kg for DEHP, confirming its strong neurotoxic potential. In contrast, DEHP showed highest sensitivity in the hippocampus (SOD EC_{50}: 91.2 mg/kg), consistent with previous reports highlighting this region's vulnerability to phthalate-induced oxidative stress and developmental neurotoxicity[15] [16]. These patterns provide mechanistic insight into differential regional susceptibility and potential interaction effects under combined exposure.

Maternal Endocrine Disruption

Perinatal exposure to DEHP and cadmium significantly altered maternal physiology and endocrine homeostasis during late gestation (GD 19–21). Exposed dams exhibited reduced final body weight (≈14–15%) and decreased feed intake, particularly in the DEHP group, reflecting a metabolic burden likely linked to endocrine disruption[17]. Hormonal profiling indicated a decompensated hypothyroid state characterized by elevated TSH with reduced fT_3 and fT_4 levels. This disruption was most pronounced under co-exposure, where TSH levels increased more than twofold while thyroid hormones declined sharply, indicating impaired thyroid–pituitary feedback and diminished thyroidal output. Reduced thyroid hormone availability during late gestation likely contributed to the observed suppression of maternal weight gain and appetite[9] [18].

In addition, estrogen levels declined across exposure groups, with the greatest reduction observed in co-exposed dams. This finding suggests concurrent disruption of the hypothalamic–pituitary–gonadal axis and supports previous reports describing phthalate- and cadmium-induced endocrine interference[19–21]. Collectively, these findings indicate that combined exposure imposes compounded physiological stress during gestation.

Offspring Growth and Endocrine Alterations

Perinatal exposure produced toxicant-specific effects on offspring growth and endocrine regulation at weaning (PND-21). Cadmium or DEHP exposure alone caused severe growth restriction (~33–35% reduction in body weight), whereas co-exposure resulted in a comparatively attenuated deficit (~17%), suggesting a non-additive interaction possibly related to altered toxicokinetics or competitive uptake[22]. Despite reduced body weight, individually exposed offspring exhibited an increased brain organosomatic index, indicating preferential preservation of brain mass. This pattern is consistent with the brain-sparing response described within the Developmental Origins of Health and Disease (DOHaD) framework, where early-life stress prioritizes neural development over peripheral growth[6].

Endocrine profiling further revealed distinct thyroid signatures among exposure groups. Cadmium exposure produced a hypothyroid-like pattern (elevated TSH with reduced fT_3 and fT_4), consistent with metal-induced disruption of thyroid hormone synthesis and signaling[23]. In contrast, DEHP exposure was associated with suppressed TSH and elevated thyroid hormone levels, suggesting altered hypothalamic–pituitary regulation, as previously reported in phthalate exposure studies[9,18]. Co-exposed offspring exhibited a dissociation between fT_4 and fT_3 levels, with elevated T_4 but markedly reduced T_3, indicating impaired peripheral deiodination. Given the critical role of T_3 in brain maturation and metabolic regulation, this selective disruption represents a key mechanistic pathway underlying combined DEHP–cadmium toxicity[24].

Maternal–Offspring Endocrine Divergence

A striking divergence emerged between maternal and offspring endocrine profiles. While exposed dams displayed hypothyroidism, co-exposed offspring exhibited severe hypercortisolism, reflecting persistent activation of the hypothalamic–pituitary–adrenal (HPA) axis. Elevated cortisol is known to suppress pituitary TSH secretion, explaining the reduced TSH levels observed in offspring despite elevated circulating T_4. This paradoxical profile likely reflects impaired deiodinase-mediated conversion of T_4 to active T_3 and suggests stress-induced endocrine reprogramming during early development[9,18]. The previously described brain-sparing response may further interact with cortisol-driven programming, producing a stress-adaptive yet hormonally dysregulated phenotype distinct from the maternal endocrine state.

CONCLUSION

In conclusion, perinatal co-exposure to DEHP and cadmium induces stage-specific and pathway-selective endocrine and developmental toxicity that cannot be predicted from single-chemical studies alone. Integration of EC_{50}-based dose–response modeling with maternal and offspring endocrine outcomes revealed convergent disruption of thyroid, metabolic, and growth-regulatory pathways, with unique signatures emerging under combined exposure. These findings underscore the importance of mixture-based risk assessment in developmental toxicology and highlight the potential public health risks posed by real-world environmental chemical mixtures

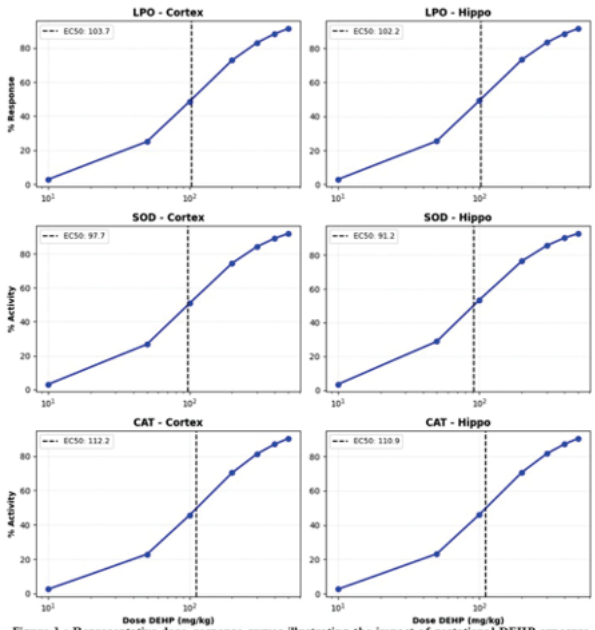
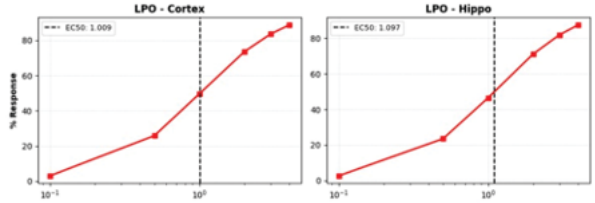


Figure 1 : Representative dose–response curves illustrating the impact of gestational DEHP exposure on oxidative stress biomarkers in the cortex and hippocampus. Sigmoidal profiles for lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT) were derived using a four-parameter logistic nonlinear regression model to establish biologically relevant EC50 values. Vertical dashed lines indicate the specific EC50 thresholds (ranging from 91.2 to 112.2 mg/kg) that served as the basis for dose selection in the subsequent Phase II interaction study. All data points represent the mean percentage of the maximal biological response or activity (n=6 animals per dose group) across the cumulative exposure range.



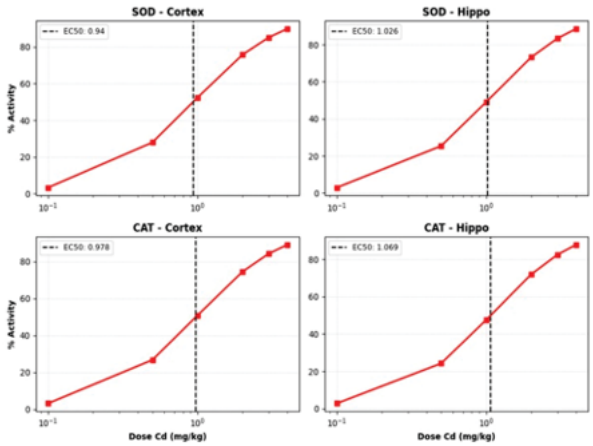


Figure 2 : Representative dose–response curves illustrating the impact of gestational Cadmium exposure on oxidative stress biomarkers in the cortex and hippocampus. Sigmoidal profiles for lipid peroxidation (LPO), superoxide dismutase (SOD), and catalase (CAT) were derived using a four-parameter logistic nonlinear regression model to establish biologically relevant EC50 values. Vertical dashed lines indicate the specific EC50 thresholds (ranging from 91.2 to 112.2 mg/kg) that served as the basis for dose selection in the subsequent Phase II interaction study. All data points represent the mean percentage of the maximal biological response or activity (n=6 animals per dose group) across the cumulative exposure range.

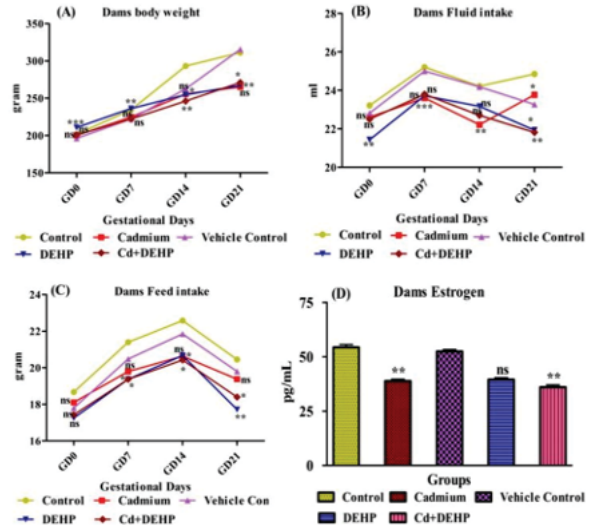


Figure 3: Representative bar graphs illustrating alterations in gestational rats (GD-19) following different experimental exposures. (A) Dams body weight, (B) Feed intake, (C) Fluid intake, and (D) Estrogen. The experimental groups include Control, Vehicle Control (olive oil), Cadmium (Cd), Di (2-ehtylhexyl) phthalate (DEHP), and combined DEHP+Cd exposure administered throughout the gestational period. Data are expressed as Mean±SEM (n=6 animals/group). Symbol '*' represents significantly different as * p < 0.05, ** p < 0.01, *** p < 0.001 and 'ns' non-significant from their corresponding controls as determined by Dunn's post hoc test.

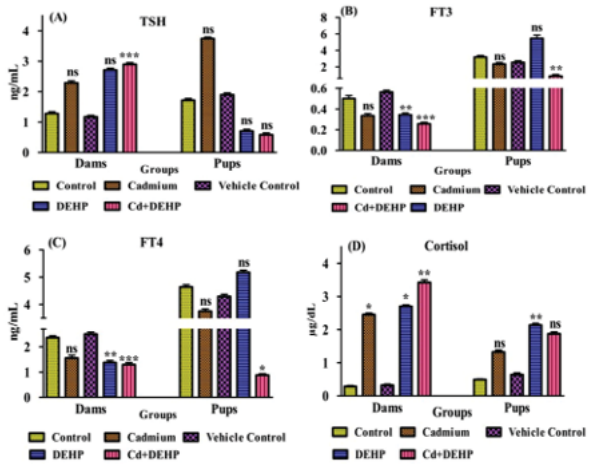


Figure 5: Representative bar graphs illustrating alterations in serum hormone levels in gestational rats (GD-19) and their offspring (PND-22) following different experimental exposures. (A) Thyroid-stimulating hormone (TSH), (B) Triiodothyronine (T3), (C) Thyroxine (T4), and (D) Cortisol. The experimental groups include Control, Vehicle Control (olive oil), Cadmium (Cd), Di (2-ehtylhexyl) phthalate (DEHP), and combined DEHP+Cd exposure administered from gestation through lactation period. Data are expressed as Mean±SEM (n=6 animals/group). Symbol '*' represents significantly different as * p < 0.05, ** p < 0.01, *** p < 0.001 and 'ns' non-significant from their corresponding controls as determined by Dunn's post hoc test.

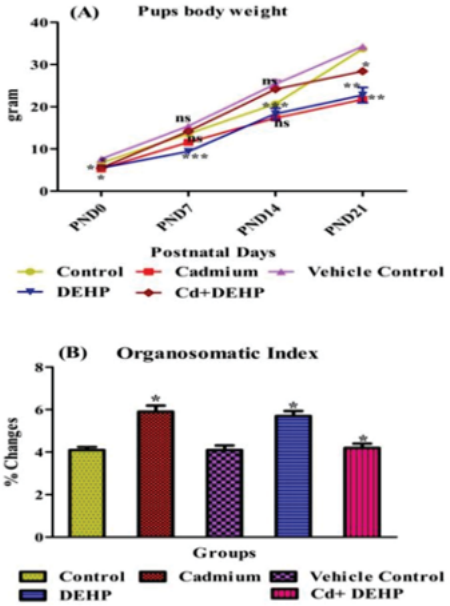


Figure 4: Representative bar graphs illustrating alterations in gestational rats (GD-19) following different experimental exposures. (A) Pups body weight , and (B) Brain Organosomatic index. The experimental groups include Control, Vehicle Control (olive oil), Cadmium (Cd), Di (2-ehtylhexyl) phthalate (DEHP), and combined DEHP+Cd exposure administered from gestation through lactation period. Data are expressed as Mean±SEM (n=6 animals/group). Symbol '*' represents significantly different as * p < 0.05, ** p < 0.01, *** p < 0.001 and 'ns' non-significant from their corresponding controls as determined by Dunn's post hoc test.

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