HUMAN DOSES OF DEHP DURING PREGNANCY INFLUENCE POST-NATAL GROWTH AND REPRODUCTION IN OFF-SPRING FEMALE MICE

A THESIS

BY

HASAN-UZ-ZAMAN

Student No. 1504096

Semester: July-December, 2023

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MASTER OF SCIENCE (M.S.)

IN

GENETICS AND ANIMAL BREEDING

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DEPARTMENT OF GENETICS AND ANIMAL BREEDING

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY

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Dedicated To My
Beloved Parents and
Honorable Teachers

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is an endocrine-disrupting chemical that interferes with reproductive function and hormone regulation. Among different exposure routes (oral ingestion, inhalation, and dermal contact), the most common routes of exposure are via oral ingestion from food packaging and the use of cosmetic products, but high levels of phthalates are also present in household dust. Thus, exposure to phthalates is ubiquitous in human populations. For the general population, it was estimated that the average total daily individual ambient exposure to DEHP ranges from 0.21 to 2.1 mg/day. Thus, the estimated range of daily human exposure to DEHP is 3–30 μg/kg/day based on urinary metabolite concentrations; however, measurements of DEHP in household dust can reach up to 700 mg/kg, potentially increasing exposure levels in certain individuals. The effect of phthalate on male reproduction is now well studied; however, the effect of phthalate on female reproduction warrants extensive investigation. Because women are exposed to phthalates at a higher level than men through their widespread use of personal care and cosmetic products, In addition, it is hypothesized that phthalate can cross the placenta and interfere with the embryo, postnatal growth and development, and the later life of offspring after parturition. In this study, the Swiss albino female mice were randomly divided into four groups namely T0 (control), T1 (2mg/kg DEHP), T2 (4mg/kg DEHP), and T3 (6 mg/kg DEHP). Mating's strategy was followed by two virgin females and one stud male, who were confirmed pregnant by observing a vaginal plug. DEHP was exposed from the plug confirmed to parturition. After parturition, litter size (number of pups), litter weight, weight/pups, and body weight took until sexual maturity, then evaluated the reproductive cyclicity, the hormonal profile, hematological parameters, and organ developments after sexual maturity of female mice.

The result showed that from birth to weaning, offspring of the T1 group exhibited a significantly body weight gain compare to the T0 group. On the other hand, considering weaning to sexual maturity, the rate of body weight gain was higher in the T1 and T2 groups

compared to the T0 group. The final body weight was higher in the T2 group compared to the offspring of the T0 group. The estrous cyclicity data revealed that the rate of estrous in the T1, T2, and T3 groups were gradually decreased compared to the T0 group, in case of proestrus T1 was increased compared to T0 group but T2, T3 group were decreased compared to T0 group. The rate of diestrus in the T1, T2 and T3 groups were increased compare to the T0 group. On the other hand the rate of metestrus in the T1 and T2 group were decreased compare to T0 group but T3 was increased compared to T0 group. In the hormonal assay, the percentage of estrogen in the T1, T2 and T3 were gradually decreased compared to T0 group and the percentages of progesterone levels in the treatment groups were decreased significantly. Hematological parameters showed that the platelets in the T1, T2, and T3 groups were significantly higher than T0 group. In the term of neutrophil percentage, the T3 group was significantly lower than the other groups, Lymphocyte percentage in the T1, T2, and T3 groups were significantly higher than the T0 group. In case of total WBC count in the T1, T2 and T3 groups were increased compared to T0 group. The hematological parameters of PCT, RDW-CV, and RDW-SD were significantly lower in the T1, T2, and T3 groups than the T0 group. The percentage of monocytes in the T1, T2 and T3 were gradually decreased compared to T0 group. In cases of organ development, lung weight in the T1 and T3 groups were significantly increased from the T0 group but the T2 group was decreased from T0 group. In the T1, T2 and T3 groups, kidney and liver weight were significantly higher than in the T0 group. Heart weight in the T2 and T3 groups were significantly higher than in the T0 group. In the same way, spleen weight is comparatively higher in the T1 and T2 groups compared to the T0 group. There were no significant changes in the thyroid, uterus, or ovaries. Visual observation of the morphological characteristics of different organs showed that slight discoloration of the liver and lung was substantially affected in T3 group compared to T0 group.

Keywords: Phthalates, Body Growth, Organ development, Organ morphology, Blood analysis, Biochemical assay, Hormonal assay

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ABBREVIATION AND ACRONYMS

DEHP	Di (2- ethylhexyl) phthalate
PVC	Polyvinyl Chloride
LDPE	Low Density Polyethylene
DNA	Deoxyribonucleic acid
GI	Gastrointestinal
E2	Estradiol
MEHP	Mono (2-ethylhexyl) Phthalate
PND	Postnatal Days
F1	First generation
Icddr, b	International Centre for Diarrheal Disease Research, Bangladesh
ARF	Animal Resources Facility
G	Gram
ml	Milliliter
ANOVA	Analysis of Variance
%	Percentage
g/dl	gram per deciliter
SEM	Standard error mean

CHAPTER 1

INTRODUCTION

Phthalates are diesters of phthalic acid (1,2-benzenedicarboxylic acid) and are synthetic organic chemicals used in industries as solvents, plasticizers, and additives in polyvinyl chloride (PVC) plastics or personal care products (PCPs) [Latini *et al*., 2005]. There are currently around 25 different phthalates in use in commercial applications, and each one gives a certain quality to the product it is added to. Ten phthalates that are frequently used are dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), diisobutyl phthalate (DiBP), benzyl butyl phthalate (BzBP), dicyclohexyl phthalate (DCHP), di(2-ethylhexyl) phthalate (DEHP), di-n-octyl phthalate (DnOP), di-isononyl phthalate (DiNP), and di-isodecyl phthalate (DiDP). One of the most common phthalates in trade, DEHP, was initially created in 1933 as a plasticizer [Frederiksen *et al*., 2007]. Phthalates have gained global popularity due to the use of DEHP as an addition to polyvinyl chloride (PVC) to increase plastic's flexibility. Phthalates are added to PVC to provide it flexibility, malleability, and durability. Up to 50% (by weight) of phthalates can be found in PVC goods [Latini *et al*., 2005]. DEHP is among the most harmful chemical additives in plastic for human health. According to many studies and reviews, these include the possibility of an impact from recycling processes (Geueke *et al*., 2018), the frequency of use in primary plastic products (Groh *et al*., 2019), and the human health hazard score (Hahladakis *et al*., 2018). Because of their widespread usage in plastics and their provenance as endocrine and metabolic disruptors, this class of compounds has drawn a lot of media attention. International research consortiums are also bringing these challenges to light. In a recent summary of the widespread health effects of various endocrine-disrupting chemicals found in plastics, including DEHP, the Endocrine Society and the International Pollutants Elimination

Network (IPEN) acknowledged the growing concern surrounding these chemicals in the context of the circular economy (Flaws *et al*., 2020).These days, DEHP may be found in a wide range of items, including construction supplies, car components, food packaging, toys, building adhesives, paints, flooring, lubricants, hair sprays, shampoos, soaps, nail polishes, and detergents. [Graham 1973]. In 2006, 4.7 million metric tons of DEHP were produced worldwide [Mackintosh *et al*., 2006; Sirivarasai *et al*., 2013], and in 2015, ~8 million metric tons [Sempere *et al*., 2015]. DEHP, DiNP and BzBP are additives found in most commercial items; they readily move into the environment by abrasion, leaching, and evaporation [Gimeno *et al*., 2014]. DEHP are pervasive environmental pollutants detected in various environmental matrices, such as sludge, dust, soil, air, and water [Wormuth *et al*., 2006]. Both people and animals are predominantly exposed to DEHP through oral means. Eating food, especially food from containers that leak DEHP, accounts for around 95% of total oral exposure in adults and children; dust inhalation accounts for the remaining 5% (Clark *et al*., 2011). For toddlers and newborns, the total oral intake is about equal to the amount ingested by food and dust particles containing dioctyl phthalate (Clark *et al*., 2011; Wormuth *et al*., 2006). According to Petersen and Breindahl (2000), DEHPs are readily broken down and can pollute the environment since they do not create a strong connection with the polymer. Because of this, exposure occurs to both people and animals by eating, breathing, or skin absorption. The acceptable daily consumption of DEHP for humans is 20 µg/kg/bw/day, whereas the reference dosage is 50 µg/kg/bw/day (Przybyli *et al*., 2016). The average daily ambient exposure to DEHP in the general population was estimated to be between 0.21 and 2.1 mg/day (David *et al*., 2000; Doull *et al*., 1999; Huber *et al*., 1996). Based on urinary metabolite concentrations, the estimated range of daily human exposure to DEHP is therefore 3–30 µg/kg/day; however, DEHP levels in home dust have been measured to reach up to 700 mg/kg, which may increase exposure levels in certain persons (Becker *et al*. 2004). It is

hardly unexpected that those who are exposed to DEHP at work have significantly greater amounts. For instance, according to estimates (Ruder *et al*., 1990), approximately 340,000 and 239,149 workers, respectively, were exposed to DEHP and DEP throughout the 1980s, with DEHP ranging from 143 to 286 μg/kg/day. Furthermore, Tabacova *et al*. (1982) found a correlation between elevated Proposal Format-IRT 4 urine phthalate levels and pregnancy problems in women, including anemia, toxemia, and preeclampsia. DEHP lowers implantations, raises resorptions, lowers offspring fetal weights, and lowers the incidence of pregnancy in experimental animals (Kaul). DEHP is thought to have the potential to penetrate the placenta and negatively impact the developing embryo, the postnatal period, the offspring's later life, and their development. The most popular and concerning is DEHP, which has been found in research to operate as an endocrine disruptor and have negative effects on both people and lab animals (Huang *et al*., 2022). The appropriate synthesis of sex hormones like progesterone and androgens is adversely affected by endocrine disruptors, which have been demonstrated to selectively target receptors in the hypothalamic-pituitary-gonadal system. Consequently, they can affect regular hormonal processes by attaching to steroid hormone receptors and triggering the cell that carries the receptor to respond (Csaba, 2018). A negative correlation has been found between insulin resistance syndrome and endocrine-disrupting substances, as demonstrated by the worse prognoses of those with greater exposure levels. Additionally, they change the way sex hormones are produced, which has a detrimental effect on both adult sexual behavior and children's sexual development. Endocrine disruptors can have a direct impact on the development of the sex organs as teratogens during fetal life (hypospadias, macropains, cryptorchidism) and as improper imprinting during late sexual events (pubertal, adult) (functional teratogens, when exposure occurs perinatally or later) (Hauser *et al*., 2016).

Limited data in animal studies indicate that in oral animal trials, effects are frequently observed at modest doses, including changed immunological responses, harm to the sexually mature male and female reproductive systems, folliculogenesis, altered renal effects, altered hepatic effects, and hormonal disturbance. In this research, pregnant mice will be exposed to human doses of DEHP to evaluate the ovarian function of offspring mice. To address the issue, the research has the following specific objectives:

1. Observation of reproductive cyclicity (estrous cycle) in offspring mice
2. Assessment of ovarian hormone production in offspring mice
3. Evaluation of organ developments in offspring mice after sexual maturity

CHAPTER 2

REVIEW OF LITERATURE

The chapter reviews the research on the effects on postnatal growth, blood parameters, and organ development in offspring female mice exposed to DEHP. To understand and internalize every learning issue related to the current study, a review of the literature was conducted on the following topics: the physical and chemical properties of phthalates; uses of phthalates; sources of phthalates; routes of phthalates; phthalates contamination in oral exposure on a global basis as well as Bangladesh; metabolism and distribution of phthalates; toxic effects of phthalates in human and animal health; effects of phthalates on offspring body weight and internal organs; and hematological and hormonal assessment.

2.1. Physical and Chemical Properties of DEHP

Properties	Information
Chemical name	Di-2-ethylhexyl phthalate (DEHP)
Synonym	DEHP; DOP; bis(2-ethylhexyl) phthalate; Bisoflex 81; Eviplast 80; Octoil; Plantinol DOP; Staflex DOP; 1,2-benzenedicarboxylic acid, 1,2-bis(2ethylhexyl) ester
Chemical formula	C24H38O4
Molecular weight	390.56
Appearance (clarity)	Clear
Color	Colorless
Physical state	Liquid
Solubility	Weak solubility in water and satisfactory solubility in most organic solvents.
Boiling point	384^{o}c
Melting point	-55^{o}c
Density at 20°C	0.984 g/cm

2.2. Uses of DEHP

DEHP has been used for more than 50 years in a variety of consumer and industrial uses. It has many different qualities for a variety of uses, and depending on its molecular weight, it may be divided into two categories: It is commonly known that phthalates, both long and short chain, as well as high molecular weight phthalates, such as butyl benzyl phthalate (BBzP), di-2-ethylhexyl phthalate (DEHP), and mixes of di-n-octyl phthalates (DnOP), are used as plasticizers in polyvinyl chloride (PVC) materials, which include food packaging, apparel, flooring, and medical equipment. Over the past few years, DEHP has gradually been replaced in these applications by didecyl phthalate (DiDP) and di-nonyl phthalate (DiNP) (Zota *et al*., 2014). Conversely, low-molecular-weight phthalates such as dimethyl phthalate (DMP), diethyl phthalate (DEP), and dibutyl phthalate (DBP) are widely utilized in cosmetics and personal hygiene products as solvents, fixatives, and adhesives (Satyanarayana *et al*., 2010). The phthalate compounds' non-covalent bonds with their parent materials have the potential to generate substantial leaching and volatilization, contaminating the environment and exposing the population to frequent exposures. DEHP is typically used to dissolve monomers so that they may more easily crosslink into polymers (like vinyl chloride into PVC) (Chaudhary *et al*., 2016). When the plasticizer is retained throughout the polymerization process, it contributes to the plastic's increased flexibility, decreased hardness, and decreased tensile strength (Chaudhary *et al*., 2016). DEHP-containing plastics are present in a wide range of everyday objects, including wall coverings, tablecloths, floor tiles, shower curtains, furniture upholstery, garden hoses, swimming pool liners, rain gear, baby pants, dolls, toys, shoes, car tops, packaging film sheets, medical tubing, sheathing for wire and cable, and blood storage bags.

2.3. Sources of DEHP in Dietary Products

Dicyclohexyl phthalate (DCHP) had a detection frequency range of 6%, whereas di-2-ethylhexyl phthalate (DEHP) had a detection frequency range of 74%. Pork had the highest estimated mean concentration of any food category (mean 300 ng/g; maximum 1,158 ng/g), with DEHP concentrations being the highest of the phthalates assessed in all foods with the exception of beef, where di-n-octyl phthalate (DnOP) was the highest phthalate observed. For dimethyl phthalate (DMP), the estimated mean adult intakes varied from 0.004 μg/kg/day to 0.673 μg/kg/day. (Arnold and others, 2013) Meat and dairy products include greater amounts of plasticizers than cereals, vegetables, and fruits. This might be because these chemicals were more easily deposited in high-fat diets. Studies show that bread and high-fat dairy items like cheese and butter, which contain a lot of plasticizers, are the main sources of plasticizer exposure. Based on the estimated daily intakes of di-2-ethylhexyl phthalate (DEHP) of US women of reproductive age, adolescents, and infants for typical consumption patterns as well as healthy and poor diets, there may be a relationship between these food groups and the degree of food processing (Y-I-Chen *et al*., 2021). Throughout food monitoring experiments, we routinely found high DEHP concentrations (≥ 300μg/kg) in chicken, cooking oils, and cream-based dairy products. Positive correlations between DEHP and the consumption of meat, dairy products, and discretionary fat were found in epidemiological research. Based on average diets, the DEHP expose Dicyclohexyl phthalate (DCHP) had a detection frequency range of 6%, whereas di-2-ethylhexyl phthalate (DEHP) had a detection frequency range of 74%. Pork had the highest estimated mean concentration of any food category (mean 300 ng/g; maximum 1,158 ng/g), with DEHP concentrations being the highest of the phthalates assessed in all foods with the exception of beef, where di-n-octyl phthalate (DnOP) was the highest phthalate observed. For dimethyl phthalate (DMP), the estimated mean adult intakes varied from 0.004 μg/kg/day to 0.673 μg/kg/day. (Arnold and others, 2013) Meat and dairy

products include greater amounts of plasticizers than cereals, vegetables, and fruits. This might be because these chemicals are more easily deposited in high-fat diets. Studies show that bread and high-fat dairy items like cheese and butter, which contain a lot of plasticizers, are the main sources of plasticizer exposure. Based on the estimated daily intakes of di-2-ethylhexyl phthalate (DEHP) of US women of reproductive age, adolescents, and infants for typical consumption patterns as well as healthy and poor diets, there may be a relationship between these food groups and the degree of food processing (Y-I-Chen *et al*., 2021). Throughout food monitoring experiments, we routinely found high DEHP concentrations (≥ 300μg/kg) in chicken, cooking oils, and cream-based dairy products. Positive correlations between DEHP and the consumption of meat, dairy products, and discretionary fat were found in epidemiological research. Based on average diets, the DEHP exposure estimates for women of reproductive age, adolescents, and babies were 5.7, 8.1, and 42.1μg/kg-day, respectively, with dairy being the biggest contributor to exposure. Exposure increased by twofold in diets heavy in meat and dairy products. The reference dosage of 20μg/kg-day set by the Environmental Protection Agency was surpassed by estimates for newborns based on a normal diet, and this threshold was also exceeded by diets heavy in dairy and meat consumed by teenagers. The literature study revealed that DEHP is regularly present in high amounts in some meats, fats, and dairy products and can lead to exposure (Serrano *et al.*, 2014).

2.3.1. Medical Purposes

Medical equipment made of polyvinyl chloride that is used to give IV fluids, blood, nutritional formulas, and breathing gasses that have been weakened with DEHP can leak phthalates at different rates. Solutions containing lipids facilitate leaching. A maximum daily DEHP exposure of approximately 9.5 mg/day, or 0.14 mg/kg/day, is thought to result from an internal formula containing lipid emulsion that is stored in a polyvinylchloride (PVC)/DEHP

bag and administered through PVC/DEHP tubing; neonatal infants may be exposed to 2.5 mg/kg/day via this pathway (FDA, 2001). Although there hasn't been much research done on exposure quantification, breathing gasses that go through PVC tubes may include DEHP. Based on a direct measurement conducted in an experiment, the estimated daily exposure to DEHP during respiratory treatment is 28.4–94.6 mg (Ted Schettler *et al*. 2005). Different phthalates have been detected in medicines across a wide variety of therapeutic classes, including analgesic, antibiotic, GI, cardiovascular and antiepileptic medication products. As expected, we found that the most common use of phthalates was in prescription medicine formulations that provided a controlled and timed release of active ingredients. Phthalates have been found in a variety of non-prescription medicine and supplement formulations for enteric coatings, film coatings, and specialty capsule formulations for various product categories and uses. These included reducing the aftertaste from fish oil and garlic, minimizing the upset stomach caused by iron and laxatives, minimizing the upset stomach caused by probiotics and acid-reducing medications, and developing soft gelatin capsules (also called "soft gels") that may be easier to swallow or contain solubilized medications and oils (e.g., ibuprofen, fish oil) (Katherine E. Kelley *et al*., 2012).

2.3.2. Care and Cosmetics

Phthalates are multipurpose chemicals that are present in a wide range of consumer products, such as makeup and personal hygiene products. Skin cleansers, nail polish, hair care products (hair sprays, mousses, and gels), body lotions (body lotions and body creams), deodorants (including antiperspirants), and baby products (oils, lotions, shampoos, and diaper creams) were among these goods. Samples were extracted using different organic solvents based on the kind of products, and gas chromatography-mass spectrometry (GC-MS) was used for analysis. The highest daily exposure to DEHP for adult females was 0.82μg/kg bw/d (Diane

Koniecki panel, 2011). DEHP concentrations were much higher in dorms where bottled skincare items were often used (Adibi *et al*., 2008) found statistically significant correlations between personal air concentrations of DEP, DnBP, and BBzP and urinary levels of the respective monoester metabolites in 25 pregnant women from New York. No such correlation was found for DEHP (Becker *et al.*, 2004) Investigated the association between urinary DEHP metabolite levels in 254 German children and DEHP concentrations in house dust samples from their homes. Despite the high phthalate contamination levels found in the house dust studies, there was no discernible correlation with the body load as assessed by urine phthalate metabolite monitoring. Thus, food is the primary source of exposure to long-chain phthalates like DEHP and DiNP, while other sources are equally or even more significant for short-chain phthalates like DnBP, DiBP and BBzP. This is supported by data from Koch *et al*. (2005) and other studies.

2.4. Routes of DEHP Exposure

Phthalates' physicochemical characteristics differ based on their chemical structure, even though their vapor pressures are usually not very high. Lipophilicity is a characteristic of phthalates that influences their environmental leaching and partitioning. The following are possible exposure routes: intravenous injection, ingestion, inhalation, and skin absorption. Humans and animals may be exposed to phthalates by direct use or contact with phthalate-containing products, through product-to-product leaching (e.g., phthalate-contaminated food packaging, intravenous fluids), or general ambient contamination.

2.4.1. DEHP Contamination in Oral Exposure on a Global Basis

Foodstuffs such as internal nutritional formulae, medications, nutritional supplements, children's toys that need sucking and other mouthing items can all contain phthalates. Foods that have been repeatedly reported to contain significant levels of phthalate Meats: chicken

The main foods that the investigators examined were beef, chicken, and pork, both separately and in combination with other meats. All phthalate species were found in poultry. Concentrations of other phthalates were typically modest, however almost half of mean DEHP readings were higher than 300 μg/kg. The DEHP concentration of beef varied compared to other meats; it ranged from the detection limit in samples from the United States to 1100 μg/kg in Canada (Schecter *et al.*, 2013). In every evaluated study but one, it was discovered that the pork had measurable amounts of DEHP, with some values coming close to the 300μg/kg threshold. The majority of average DEHP concentrations (175.8–758.3μg/kg) approached high levels when meat products (beef, chicken, pork, and other meats) were evaluated together. Lower amounts of every other type of phthalate were reported. Fascinatingly, a Canadian investigation found that while no phthalate species were found in the frozen items, substantial DEHP concentrations were found in packed meat products that were not frozen (Lacroix GM 1998). Significant DEHP concentrations were found between 413.1 and 1300μg/kg in the absence of DEP, DMP, DnOP, DiNP or DiDP in any of the milk samples. The levels of all other phthalates were reported to be lower. When compared to other dairy products, measurements for cheese and ice cream were almost at (and sometimes beyond) the 300 g/kg threshold. Compared to yogurt, cream, and cheese had higher levels of contamination throughout the investigations including dairy products. Compared to other meats, poultry consistently exhibited a greater phthalate concentration (Serrano *et al*., 2014). According to the study, areas with metal pipes had lower mean DEHP concentrations than sample sites with plastic pipes. DEHP was found to have the highest level of phthalate pollution (606.89 ng/l) (Abdolahnejad, 2019). Every year, the worldwide market for water bottling expands, and the discovery of di (2-ethylhexyl) phthalate (DEHP) in commercial water bottles has sparked discussions and worries about potential health consequences (Dada *et al*. 2018; Pinsrithong & Bunkoed 2018; Karayaka *et al*. 2019; Wu *et al*. 2019).

2.4.2. DEHP Contamination in Oral Exposure in Bangladesh

Bangladesh produces 4,000 tons of single-use plastic each year. In Old Dhaka alone, some 250 tons of non-recyclable goods, like as straws and plastic cutlery, are sold each month. Analysts claim that Bangladesh generates 312 tons of single-use plastic every month, putting the nation's health and ecology in jeopardy. The "Stop using single-use plastic to protect human health and the environment" research by the Environment and Social Development Organization (Esdo) estimates that 3,744 tons of single-use plastics are produced nationwide year. In Old Dhaka alone, some 250 tons of non-recyclable goods, like as straws and plastic cutlery, are sold each month. Eighty to eighty-five percent of all plastics created are discarded after only one usage and end up in rivers, canals, and sewers and rivers, where they cause severe pollution that finally makes its way to the Bay of Bengal. Five firms produce 97.5 million products weighing a combined 195 tons every month, according to the report. The secretary general of Esdo, Dr. Shahriar Hossain, stated that foamed plastic contains phthalates, which are very hazardous and carcinogenic. "Our only use for single-use plastic is comfort. We should make this plastic illegal. Glass and bamboo are two other environmentally friendly materials that may be used.

2.5. Metabolism and Distribution of DEHP

The most prevalent method that DEHP is introduced to humans and animals is through oral intake. Due to its lipophilic nature, DEHP may enter the skin and lungs of both humans and mice. However, the amount of absorption is at its highest after oral dosing. Phthalates are rapidly broken down and eliminated in the urine and feces following exposure and absorption. Although phase I hydrolysis and phase II conjugation are the usual procedures for phthalates, different phthalates may undergo different metabolic processes (Frederiksen *et al*. 2007). Phase I is the hydrolysis of the phthalate diester into the perhaps more bioactive

monoester metabolite. Esterase and lipases then disseminate this metabolite systemically throughout the liver, circulation, intestinal epithelium, and other organs (Calafat *et al*. 2006a). Numerous lipases and another carboxy esterase may be found in DEHP hydrolases. DEHP hydrolase activity has been discovered in several tissues, including the pancreas, intestinal mucosa, liver, kidneys, lungs, skin, testes, and plasma (Hopf *et al*. 2014; Ozaki *et al*. 2017). While pancreatic tissue is the main source of DEHP hydrolase activity, adipose tissue contains a comparatively low level of it. Pancreatic lipases are released into the small intestine and contribute DEHP hydrolase activity to the intestinal contents. The monoester metabolites come next. 1) Generate glucuronide-conjugated monoesters that are eliminated in the urine through phase II biotransformation processes that are mediated by UGTs (uridine 5'-diphosphate glucuronosyltransferases); 2) Go through phase I biotransformation reactions, such as oxidation, to create more hydrophilic (and probably less bioactive) secondary oxidized metabolites before glucuronidation (Barr *et al*., 2003); and/or 3) A portion of the unconjugated (free) monoester and/or secondary metabolites may also be directly excreted in the urine.

2.6. Toxic Effect of DEHP on Human and Animal Health

Phthalates have been connected to anomalies in male and female fertility, cancer, testicular dysgenesis syndrome, and alterations in puberty. Phthalates have the hormonal ability to change pituitary, hypothalamus, and peripheral hormone release. According to Henrieta *et al*. (2020), phthalates can interfere with nuclear receptors, membrane receptors, intracellular signaling pathways, and the expression of genes related to reproduction within cells. Human epidemiological studies have shown a substantial correlation between phthalate exposure and poor reproductive outcomes in men and women, type II diabetes and insulin resistance, obesity and overweight, allergies, and asthma. (Yu Wang and others, 2019). DEHP has been

demonstrated to alter immune system function, thyroid signaling, liver structure and function, metabolic balance, obesity, diabetes, and breast cancer. Phthalates can cause follicle death, increase oxidative stress, and change the pattern of follicle formation. These consequences might lead to an earlier reproductive scene, infertility, and a faster decrease of ovarian reserve (Panagiotou, 2021). Phthalates may have a substantial effect on fetal implantation, development, and parturition in mouse models by interfering with the levels, signaling, and/or activity of sex hormones (Craig Z.R *et al*., 2011). (Robins J.C. *et al*., 2011). Research on humans has shown that exposure to phthalates affects reproductive hormone levels in adults, even though exposure to phthalates during in-utero development has been connected to changes in hormone levels in neonates (Mathieu-Denon Court *et al* 2015). Ovarian follicle populations, hormone levels, pup sex ratio, pregnancy loss, estrous cyclicity, and fertility were all affected by DEHP. These findings demonstrate the long-term effects of short-term adult exposure to DEHP (Chiang C *et al*., 2020).

2.6.1. DEHP and Ovarian toxicity

The ovaries, an essential primary reproductive organ, are in charge of both the generation of female gametes and the release of sex hormones. Functional abnormalities of the ovaries can cause a variety of reproductive problems, such as anovulation, aberrant secretion of estrogen, and sterility (Li L. *et al*., 2016). The ovary may be impacted by MEHP, a metabolite of DEHP. The ovarian reserves known as female antral follicles are important sources of sex steroid hormones, and the growth of these follicles depends on estradiol (E2). DEHP affects follicle growth in vitro by lowering E2 levels (Kalo *et al*., 2015). The gene that produces the enzyme that converts testosterone into estradiol, aromatase, can be expressed differently in response to estrogen. Thus, there may be a decrease in gene expression can result in lower blood E2 levels (Gupta *et al*., 2012). Mice treated with DEHP at a dosage of 20 g/kg/day–750

mg/kg/day had altered estrous cyclicity and accelerated primordial follicle recruitment due to dysregulation of ovarian mRNA and altered levels of proteins in the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which is linked to early folliculogenesis. After being exposed to DEHP, the percentage of primordial follicles decreased. At modest concentrations, DEHP can interfere with normal reproductive processes (Hannon *et al*., 2014). DEHP was converted to MEHP, which resulted in a drop in steroidogenic enzyme levels, which in turn led to a decrease in testosterone, estrone and E2 levels, according to in vitro experiments on neonatal ovaries from mice exposed to DEHP (0.2–20 μg/ml) (Gupta *et al*., 2012). Oral administration of DEHP and its metabolite MEHP has been shown to have negative impacts on oocyte meiotic maturation and development and, as a result, alter fertility (Absalam *et al*., 2016). Pregnant mice exposed to 40 μg/kg/day of DEHP during their gestation demonstrated abnormal oocyte formation (Zhang *et al*., 2014). According to Hannon *et al*. (2014), oral exposure to DEHP for 10 and 30 days at 20 μg/kg/day-750 mg/kg/day speeds up the recruitment of primordial follicles in adult mice. This is demonstrated by a decrease in primordial follicles and an increase in primary follicles. When the ovaries were evaluated on post-natal days 15 and 21, DEHP at 20–40 μg/kg/day accelerated folliculogenesis by lowering primordial follicles and increasing preantral and antral follicles after hypodermic injections during the early post-natal life (Zhang *et al* 2013). The number of primary and secondary follicles was reduced by oral gavage exposure to DEHP alone and in conjunction with benzo[a]pyrene (B[a]P), perhaps through the induction of follicular atresia in adult rats. Following exposure from hatching to maturity, DEHP in an aqueous solution at 0.1–0.5 mg/l increased the number of atretic late-stage follicles, leading to reproductive failure (Kang *et al*., 2014). The relationship between human phthalate exposure and changes in folliculogenesis has not been extensively studied. In one study, the prevalence of polycystic ovarian syndrome (PCOS), a gynecological condition frequently

linked to infertility and the appearance of big, cystic follicles that are unable to ovulate, was compared to exposure to phthalates.

2.6.2 Effect of DEHP on Body Weight and Internal organ

It was shown that the kidney and liver tissues had aberrant histological changes during the DEHP injection. Lastly, hepatic and kidney damage may arise from DEHP. (Aydemir & colleagues, 2018) The information he gathered unequivocally demonstrated that acute DEHP injection dramatically injured mice's liver and kidneys when compared to control animals. Changes in the renal and hepatic tissues as well as the disruption of biochemical parameters were confirmed by the histological investigation. The nephrotoxic and hepatotoxic effects are more evident. Beltifa and colleagues, 2018). According to Alam *et al*. (2010), phthalate exposure has been shown by epidemiological animal and molecular studies to produce testicular shrinkage in 3-week-old rats and to disrupt spermatogenesis at 500 mg/kg. According to research, pregnant rats were given DBP (100 mg/kg/day) had two main effects: (1) Severe testicular atrophy with alterations in the ratio of testicular weight to body weight; and (2) A considerable decline in the number and diameter of seminiferous tubules with age (Okayama *et al*., 2017). Litter size and pup body weight are reduced when prenatal exposure to DEHP occurs (Zhang *et al*., 2017). In the F1 generation, there was no discernible effect of DEHP exposure on body weight. While DEHP did not alter body weights at other time points in the F2 generation, it did significantly reduce body weight at PNDs 8 and 21 in comparison to the control. When compared to the control in the F1 generation, the 200 g/kg/day dosage of DEHP significantly increased ovarian and uterine weight at PND 21; however, the 20 g/kg/day and 750 mg/kg/day doses of DEHP significantly lowered ovarian weight. Following exposure to the 200 mg/kg/day dose of DEHP, uterine weight and ovarian weight increased at PND 21, albeit this rise was only slightly significant in comparison to the control. At PND

60, the F2 generation's ovarian weight decreased with a 20 g/kg/day treatment of DEHP; nevertheless, this reduction merely indicated a trend toward significance. Following exposure to the 200 g/kg/day dose of DEHP, ovarian weight at PND 60 was significantly lower than it was in the control group. The F3 generation's exposure to DEHP did not significantly affect their ovarian or uterine weight at any point in time. In comparison to the control group, the liver weight was considerably raised by the 500 mg/kg/day dosage of DEHP and significantly lowered by the 20 μg/kg/day dose of DEHP. (Rattan and others, 2018) The liver weight to body weight ratio of the rats treated with 10 mg/kg of phthalate was substantially greater than that of the other rats treated with higher doses and the control rats. Rats treated with phthalate at doses of 25 and 50 mg/kg showed a little but non-significant decrease in comparison to the control group.

2.6.3. Effect of DEHP in Hematological and Biochemical Parameters

Biochemical analysis of blood parameters is essential for assessing a person exposed to a toxin's structural and functional state. Serum biochemical parameter analysis is strongly advised in order to determine target organ toxicity, assess the general health state of animals, and find early warning signs of critical alterations in stressed organisms (Keller, 2011). In 28-day trials, oral dosages of 400 mg/kg/day exhibited no hematological effects, but both male and female mice had significantly decreased hemoglobin and hematocrit at doses of 1,209 and 2,888 mg/kg/day, respectively (Xu *et al*., 2019). They did not exhibit any changes in their whole hematological evaluations when given gavage doses up to 150 mg/kg/day on PNDs 6–96 (Kim *et al*., 2018). There have been no documented hematological alterations in nonhuman primates after oral DEHP consumption. At dosages of 500 or 1,000 mg/kg/day, respectively, exposure to DEHP for 14–28 days in a row did not result in hematological alterations in sexually immature or mature Cynomolgus monkeys (Satake *et al*., 2010). Rats

treated with 10 ppm showed a substantially higher level of liver ALT activity than rats treated with 25 mg/kg as the control group. Rats given 10 mg/kg had livers with triglyceride levels that were substantially and comparably higher than rats given 25 mg/kg, 50 mg/kg, and control. Rats treated with 25 and 50 mg/kg had considerably lower serum cholesterol levels than rats treated with 10 mg/kg and the control group (Contzen *et al*., 2016). Monkeys were exposed to 500 mg/kg/day of phthalates for four days. Drop in lymphocyte count and a statistically significant rise in neutrophils; other blood parameters do not show any statistical significance (George Pugh *et al*., 2001). Complete hematological evaluations have not changed in long-term studies at food doses up to 939 mg/kg/day in rats and 1,458 mg/kg/day in mice (David *et al*. 2000a, 2000b).

2.6.4 Effect of DEHP on Endocrine system

The most common use of DEHP is as an endocrine disruptor (ED). An external material or combination that modifies the endocrine system's function(s) and subsequently has a negative impact on an organism's overall health, as well as the health of its offspring or other populations, is known as an endocrine disrupter [Nohynek *et al.,* 2013]. Gonadotropin and sex steroid hormone expression, as well as the hypothalamus-pituitary-ovary axis, were all changed in rats by DEHP (Hirosawa *et al*., 2006; Liu *et al*., 2014). In vitro, estrogenic DEHP suppressed follicle development and decreased estrogen levels (Kalo *et al*., 2015). In rats, it also reduced the recruitment of primordial follicles (Hannon *et al*., 2014). Serum E2 was decreased by DEHP treatment (2 g/kg BW/d). Through receptor-mediated signaling pathways, its metabolites lengthened estrus cycles and inhibited the synthesis of E2 in granulosa cells (Lovekamp-Swan & Davis, 2003). According to Kim *et al*. (2018), long-term low-dose DEHP in drinking water alters tissue layer thickness, ER and PR expression, and tissue-specific ER and PR localization. Endometrial epithelial cell proliferation and shape are

changed by DEHP therapy (Somasundaram *et al*., 2016). EDCs have the potential to cause endometriosis and alter endometrial responses to steroid hormones either directly or indirectly (Rier *et al*., 2002). For 4, 7, 14, 30, and 60 days, sublethal dosages of DINP (300 ppm) and DEHP (60 ppm) were administered to male and female fish in different groups. Blood serum was drawn at the conclusion of each exposure period to measure levels of thyroid stimulating hormone (TSH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), cortisol, testosterone, and estradiol. These results were compared with those of the corresponding control groups (Revathy *et al*. 2019). DEHP treat which resulted in histopathological thyroid alterations in rats: T4 levels decreased but not T3 levels [Hinton *et al*., 1983]. On the other hand, serum T3 and T4 levels were elevated in rats intravenously administered DEHP at quantities comparable to what might leak from PVC blood bags used for human blood transfusions [Gayathri *et al.,* 1983]. The disruption of gene expression linked to the hypothalamic-pituitary-thyroid (HPT) axis indicates that DEHP is harmful to thyroid hormones in zebrafsh larvae. Triiodothyronine (T3), thyroxine (T4), and thyrotropin-releasing hormone (TRH) can all be decreased by DEHP via activating the Ras/Akt/TRHr pathway and affecting hepatic enzymes that are essential for the thyroid-disrupting effects [liu *et al* 2002]. Following the length of exposure, female rats exposed to DEHP by inhalation from post-natal days 22–41 at 25 mg/m^3 had higher blood levels of cholesterol, LH, and estrogen (Kondo *et al*., 2006). Oral gavage of DEHP for eight days at a dose of 2 g/kg/day resulted in a drop in serum estradiol levels, subsequent increases in FSH levels, and the induction of the LH surge required for ovulation (Davis *et al* 1994). Moreover, adult rats exposed to DEHP had a longer estrous cycle (Davis *et al*., 1994). According to a related investigation, adult rats' blood estradiol levels were also lowered by oral gavage of DEHP at 1000–3000 mg/kg/day (Liu *et al*., 2014). Similar to this, adult mice exposed to DEHP orally for 16 weeks at 500–2000 mg/kg/day saw a reduction in blood progesterone levels, apoptosis,

and cell cycle arrest in granulosa cells, and a prolongation of estrous cycles (Liu *et al*., 2012). Similar effects on estrous cyclicity were seen, with adult mice spending more time in the estrous stage after oral administration to DEHP for 10 and 30 days at 20 μg/kg/day–750 mg/kg/day [Hanson *et al*., 2014]. Furthermore, in adult rats, chronic DBP administration at 500–1000 mg/kg/day enhanced gestational ex vivo ovarian estradiol production and reduced gestational ex vivo ovarian progesterone production from the time of weaning through puberty, mating, and gestation (Gray *et al*., 2006). In contrast, intramuscular injections of DEHP at a dose of 25–50 mg/kg/day resulted in an increase plasma concentration of progesterone in the adult ewe (Herreros *et al*., 2013)

2.6.5 Di-(2-ethylhexyl) phthalate exposure induces female reproductive toxicity

Several studies have confirmed that the obvious toxicity of DEHP is mediated by its effects on gonadal steroidogenesis and an accompanying decrease in reproductive function and fertility. (Gray *et al*., 2009; Culty *et al*., 2008). The average exposure of DEHP in humans ranges from 3 to 30 mg/kg (body weight)/d, and occupational exposure levels have been calculated to even reach up to 300 to 600 mg/kg/d. (Brehm *et al*., 2018; Loff *et al*., 2000). Based on epidemiological data, decreased conception rates, increased miscarriage rates, decreased estrogen levels, and abnormal ovulation are associated with the occupational exposure of female workers to DEHP. Thus, the health risks of DEHP exposure in humans have attracted increasing attention. The ovary is the primary reproductive organ in the female, and it regulates female endocrinology and provides a microenvironment for follicle development (Edson *et al*., 2009). DEHP and MEHP have been shown to induce a depletion of the primordial follicles and a decrease in sex hormone production. (Hannon *et al*., 2014; Lovekamp *et al*., 2001). During the 30 days of DEHP exposure, the control, 500 and 1500 mg/kg DEHP groups exhibited similar levels of body weight gain, and thus continuous DEHP

exposure for 30 days did not alter the body weight of mice. Significantly decreased ovary organ coefficient and estradiol levels were also observed in all DEHP-treated groups. Significant differences in follicle morphology were observed in histological examinations of the ovarian tissues from mice exposed to DEHP under a light microscope. The numbers of primary, secondary and antral follicles were decreased, and the numbers of oocytes in the primary follicles were obviously decreased by the 500 and 1500 mg/kg DEHP treatments. In the groups treated with DEHP, oocyte loss occurred in the primary follicles, along with a loose structure and detachment of granular cells, wide intercellular spaces between granulosa cells and theca cells and an increase in the number of atretic follicles. Estrus was increase and metestrus/diestrus was decrease by DEHP exposure Based on these results, DEHP obviously causes female reproductive toxicity (Dai *et al*., 2015).

2.6.6 DEHP exposure in reproductive outcome

DEHP and its metabolites have been identified in a number of human tissues and fluids, including urine, serum, cord blood, breast milk, amniotic fluid, and ovarian follicular fluid (Latini *et al*., 2003). The presence of DEHP in follicular fluid indicates its ability to reach the ovary, and its presence in amniotic fluid and cord blood implies it is able to reach the fetus. The Agency for Toxic Substances and Disease Registry estimates that humans are exposed daily to DEHP between 3-30 μg/kg/day. Human exposure to DEHP is of concern because it is a known endocrine-disrupting chemical and a reproductive toxicant .Epidemiological studies have associated in-utero exposure to DEHP with reduced anogenital distance (AGD) and testosterone levels in males (Swan *et al*., 2005) and experiments in rodent models have corroborated these findings (Gray *et al*., 2000). Additionally, epidemiological studies have reported correlations between prenatal exposure to DEHP and endometriosis, precocious puberty, early pregnancy loss, preterm birth, and low birth weight, though these findings have

been inconsistent (Cobelis *et al.*, 2003). The mechanism by which phthalate exposure is associated with adverse reproductive outcomes in humans is unknown. Recent studies, however, have shown that DEHP, through its bioactive metabolite mono-(2-ethylhexyl) phthalate (MEHP), may alter peroxisome proliferator-activated receptor (PPAR) downstream activity in a number of tissues in animal models. PPARs exist in three different isoforms: α, β, and γ. The receptors are located in adipose tissue, liver cells, uterine endometrial cells and throughout the ovary (Komar 2005) Changes in activity of PPARs in these tissues have been linked with reproductive problems in animal models (Kay *et al.*, 2013) and thus, may play a role in the association between phthalate exposure and reproductive outcomes in humans.

2.6.7 Transgenerational and multigenerational effects of DEHP

Although the epigenetic toxicity in the liver is less well understood, prenatal DEHP exposure enhances epigenetic multi- and transgenerational inheritance of adult onset illness in later generations (F1-F3). From gestational day (GD) 10.5 until pups were born, CD-1 mice were prenatally exposed to 20 μg/kg/day, 200 μg/kg/day, 500 mg/kg/day, or 750 mg/kg/day DEHP. Postnatal days (PNDs) 8 and 60 were used to assess the multigenerational and transgenerational impacts of mRNA expression of epigenetic regulators in the livers of F1, F2, and F3 generation mice exposed to prenatal exposure. The global DNA methylation levels of the livers of mice exposed to DEHP were shown to have changed significantly in all three generations, with the effect being different in the F2 generation following high dosage exposure. Histopathology suggested that exposure to DEHP can cause moderate liver damage in F1 animals. DNA methyltransferase 1 (Dnmt1) expression levels were markedly altered in both the F1 and F2 generations at PND 8, indicating that Dnmt1 maintenance is important for the intergenerational effects that happen during the early embryonic stages. Furthermore, ten-eleven translocation methylcytosine (Tet) dioxygenases encoding T1 expression in all three

generations and T2 expression in F3 at PND 60 were significantly altered by DEHP exposure, suggesting their roles in triggering both multi- and transgenerational effects following DEHP exposure in mouse liver (Wen *et al.*, 2022). DEHP may have an effect on several generations at key stages of development (Stenz *et al*., 2019; Li *et al*., 2018). According to certain epidemiological research, there is a correlation between the metabolite of DEHP found in maternal urine and lower testosterone levels in boys (Araki *et al*., 2014) as well as poor neurodevelopmental outcomes in kids (Minatoya *et al*., 2018). Extensive research conducted on animals has demonstrated that exposure to DEHP affects not only one generation but also generations that were not directly exposed to the toxin. The effects of DEHP across generations are not all the same. For example, Chen *et al*. (2019) found that DEHP serves as an obesogenic in drosophila, Quinnies *et al*. (2015) found that DEHP affects stress hormones and behavior in mice, and Rattan *et al*. (2018) found that DEHP increases adult-onset reproductive failure in mice. Enzymes involved in both methylation and demethylation cause changes in DNA methylation. For instance, ten-eleven translocation (TET) helps remove methylation from DNA (Tahiliani *et al*. 2009) and DNA methyltransferase (DNMT) proteins normally create and maintain DNA methylation patterns (Lyko *et al*., 2018).

2.6.8 DEHP and Neurotoxicity

The brain has been determined to be at risk of DEHP exposure. DEHP can affect neurodevelopment and lead to teratogenic anomalies by disrupting normal fetal brain development, as DEHP can cross the placenta and enter the fetal circulation (Lin *et al*., 2015; Xu *et al*., 2017). Gestational and postnatal DEHP exposure has harmful effects on rat brain development and function [Lin *et al*., 2015]. DEHP exposure (1500 mg/kg) in utero led to a metabolic disturbance of the lipid metabolome of the fetal rat brain, which caused anomalous

brain growth (Xu *et al*., 2017). The hippocampus is a major part of the brain that plays an important role in memory and spatial navigation. Post-DEHP exposure in postnatal rats has been shown to have a harmful impact on the development of the hippocampus in males but not in females (Smith *et al*., 2014). Resistance of the female rat hippocampus to modification by DEHP is because DEHP alters the lipid profile of the hippocampus during postnatal development, leading to elevated levels of phosphatidylcholine and sphingomyelin in the hippocampus of female rats but no effect of DEHP on the abundance of phosphatidylcholine and sphingomyelin in the hippocampus of male rats. These studies suggested that upregulation of hippocampal lipids may serve a neuroprotective role in DEHP-exposed female rats (Smith *et al*., 2015). Brain-derived neurotrophic factor (BDNF) is a protein that plays a critical role in the survival of existing neurons and promotes the enhancement and differentiation of new neurons and their synapses (Huang *et al*., 2001). Low-dose DEHP exposure (10 mg/kg) has been shown to affect dorsal hippocampal BDNF expression, which is down regulated in male rats. DEHP-exposed male rats have been observed to have decreased dendritic spine density (Smith *et al*., 2014). As BDNF is important for dendritic growth and makes synaptic connections between neurons, there could be an underlying mechanism decreasing BDNF expression, which could decrease dendritic spine density directly or indirectly (Huang *et al*., 2001). In addition to rats, the neurotoxicity of DEHP has also been observed in *Caenorhabditis elegans*, a nematode. DEHP exposure can lead to an accumulation of ROS intracellularly, which causes neurotoxicity. DEHP exposure can also inhibit the expression of many genes necessary for differentiation and function of AFD sensory neurons (necessary for the thermosensory response) (Tseng *et al*., 2013). Studies on the effect of perinatal DEHP exposure on the emotional behavior of mice have shown that phosphorylation of ERK1/2 in the hippocampus of pubertal mice and adult males is inhibited and that downregulation of androgen receptors in the pubertal male hippocampus and

estrogen receptor (ER) β in pubertal females and the adult hippocampus of both sexes may be associated with anxiety- and depression-like behaviors in mice (Xu *et al*., 2013). Intrauterine and lactational exposure to DEHP at low concentrations of 50 and 200 mg/kg/d decreased the levels of the N-methyl-d-aspartic acid (NMDA) receptor subunits NR1 and NR2B in the hippocampus in offspring mice, leading to impaired spatial learning and memory (Die *et al*., 2015). In the 104-week feeding studies mentioned above, DEHP caused an increase in the mean relative brain weights in male B6C3F1 mice at the highest dose (6,000 ppm). A similar effect was observed in male and female F344 rats at the highest dose of 12,500 ppm David *et al*., 2000).

2.6.9 Litter Size and Growth

Measures of litter size evaluated in epidemiological studies of DEHP include litter length, litter weight, and head and chest circumference Findings were inconsistent among the 15 studies that met inclusion criteria Zhao *et al*. (2014) observed exposure-related increases in the odds of IUGR across tertiles of maternal urinary DEHP metabolites in a case-control study in China (42 infants with IUGR and 84 controls matched on maternal age). A relationship between lower birth weight and higher urinary levels of MEHHP and MEOHP, especially among male infants, was also observed. In contrast, Sathyanarayana *et al*. (2016b) reported increased birth weight in female infants, but not male infants, with increasing DEHP metabolite levels in maternal urine, while Zhu *et al*. (2018) reported increased birth weight and ponderal index (birth weight/birth length) in male infants, but not female infants, with increasing DEHP metabolite levels in maternal urine. Kim *et al*. (2016a) reported an increase in birth length with a corresponding decrease in ponderal index in boys, but not girls, with increasing DEHP metabolite levels in infant first urine. Numerous studies reported body weight effects in rats following developmental exposure to DEHP; however, findings are inconsistent among species, strains and studies. Following gestation-

only exposure, decreases in pup body weight ≥10% were observed in Sprague-Dawley rats at doses ≥10 mg/kg/day (Chen *et al*. 2010) and ≥37.5 mg/kg/day (Piepenbrink *et al*. (2005); however, Vo *et al*. (2009a) did not observe decreased body weights until doses of 500 mg/kg/day. Findings in Sprague-Dawley rats following gestation plus lactation exposure were more consistent with the Vo *et al*. (2009a) study, reporting no body weight changes in offspring until maternal doses ≥447 mg/kg/day (Kobayashi *et al*. 2006). Consistent with this, body weight decreases in Sprague-Dawley neonates following direct exposure on PNDs 3–7 or 3–23 were only observed at 600 mg/kg/day (Camacho *et al*. 2020). Similarly, decreased offspring body weight in Long-Evans rats was only observed at 750 mg/kg/day, not at 10 mg/kg/day (Lin *et al*. 2009). Most studies in Wistar rats also reported no changes in offspring body weight following gestational and/or lactational exposure to maternal doses up to 700 mg/kg/day (Carbone *et al*. 2010). However, a few studies reported decreased offspring weights following maternal exposure. One study reported decreased birth weights at maternal doses ≥300 mg/kg/day (Christiansen *et al*. 2010). Other studies reported decreased postnatal body weights at maternal doses ≥1 mg/kg/day (measured on PNDs 9–22; Parsanathan *et al*. 2019) or ≥10 mg/kg/day (measured on PND 80; Rajagopal *et al*. 2019a). Additionally, two very low dose studies reported decreased offspring weight, body fat percentage, and adipocyte size at maternal doses ≥0.25 mg/kg/day during gestation and lactation (Lin *et al*. 2011).

CHAPTER 3

MATERIALS AND METHODS

The present study was designed to evaluate the toxicity on body growth, estrous cyclicity and assessed hormonal and biochemical alteration and organ morphology of offspring after DEHP exposure during embryonic days 19.

3.1. Animal selection, housing, and care

According to the guidelines recommended, laboratory-bred Swiss albino mice of either sex, 6-8 weeks of age, and weighing 25-30 g were obtained from The Animal Resources Facility (ARF) of the International Centre for Diarrheal Disease Research, Bangladesh (icddr, b) at the Laboratory Animal Research Facilities of the Department of Genetics and Animal Breeding. Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur. They were kept in an animal house with plenty of ventilation. The housing was kept at a temperature of 26±2°C with a relative humidity of 30-70% and provided the animals with complete access to food and water. The animals were housed under-regulated lighting (12-hour light/dark cycle). Five mice per cage for female mice and one mouse per cage for male mice were selected randomly and housed in plastic cages with wood-cube bedding. Provide bedding that was sanitary and cozy within the cage. To provide a relaxing environment, drinking water and sleeping materials (wood-cob) were transformed every morning. They were provided regular food in the form of pellets. Water and food were always available. Every day, the amount of water consumed was recorded Different groups of animals were confined individually. Before the beginning of the research, the mice were maintained for a week to acclimate to the new surroundings and the cages were marked with the appropriate introduction all animal experiments were conducted according to the Guidelines for the Care

and Use of Laboratory Animals with the approval of the HSTU Laboratory Animal Care and Use Committee.

Photo 3.1: Daily routine work in animal house. A) Cleaning the animal cage B) Replacing the cage with fresh wood cob as bedding materials C) Supply food in pellet form D) Drinking water supply

3.2. Treatment option and DEHP exposure

Commercially available Di (2-ethylhexyl) phthalate (DEHP) will be purchased. The dose of DEHP that mimics human exposure will be formulated using the published literature. Then the further experimental doses will be initiated through Oral gavage. Briefly, Pregnant female mice were randomly divided into four experimental groups (n=6 animals per group), ie, (1) control group - animals received filtered water (2) 2mg/kg body weight DEHP exposure, this

is produced by Sigma Aldrich co (3) 4mg/kg body weight DEHP exposure, this is produced by Sigma Aldrich co, and (4) 6mg/kg body weight DEHP exposure, this is produced by Sigma Aldrich co, and the experiment will be repeated at least three times. The control group received the same volume of deionized water as the treatment group. Animals had free access to water and food before and during exposure. Food and water consumption were recorded daily in all groups throughout the experiments. The stages of the estrous cycles in each mouse will be determined by checking vaginal lavage/smear. Mating's strategy will be two virgin females and one stud male. The females will be examined each morning for the presence of a semen plug. Detection of a semen plug will be designated embryonic day 0.5 (E0.5) of gestation. Plug-positive females will be considered ''pregnant'' and immediately placed in separate cages and exposed to DEHP through oral gavage on a continuous basis throughout the gestational stages.

3.2.1 Calculation of DEHP Dose

The dose-by-factor method applies an exponent for body surface area, which account for the difference in metabolic rate, to convert doses between animals and humans. Thus, HED (Human exposure dose) is determined by the equation:

HED (mg/kg) = Animal exposure dose (mg/kg) × (Animal k_m/ Human k_m)

The correction factor (K_m) is estimated by dividing the average body weight (kg) of species to its body surface area (m^2). For example, the average human body weight is 60kg, and the body surface area is 1.62 m^2. Therefore, the K_m factor for humans is calculated by dividing 60 by 1.62, which are 37. The Km factor values of various animal species.

3.2.2 Preparation of DEHP

Phthalates are purchased from Sigma Aldrich Company that contains 0.986mg per ml.

- At first, we took the body weight of an individual mouse then we calculated the total amount of phthalates needed per animal
- Make a DMSO solution that contains 95% distilled water and 5% Ethanol
- DMSO is a chemical that increases solubility and increases the volume of solution
- We exposed DEHP individual animals on the basis of body weight in three groups

Photo 3.2: Preparation of working solution. A) Taking DEHP solution by using micro-pipette B) Withdraw working solution by feeding needle.

3.3. Breeding procedure

Female mice were randomly selected and mated with male mice from separate cages once proestrus was proven to produce time-pregnant females. This produced the FI generation.

3.3.1 Separation of the cages of breeding mice

Male and female mice were acquired, and they were reared in separate cages Male mice were housed separately (one mouse per cage) a few days before the start of mating, whereas female mice were housed in a herd (five mice per cage).

3.3.2. Getting the next generation through breeding

The male and female mice were then combined and mating, keeping the important aspects in mind

- Sexual maturity: Male and female mice reached sexual maturity at 8 weeks, at which point they could mate and give birth
- Sexual cycle. The female mouse was watched, and when proestrus was confirmed, she was picked for mating, increasing the rate of reproduction.
- Indigenous effect. The breeding rate of the male mouse rose when a female was added to the cage
- Numbers of mice. There are one to two female mice for every male mouse. (Male to Female Ratio 1:2)
- Time. In the evening, male and female mice were housed together.
- The female vulva was inspected the next morning, and if the vaginal plug was seen, mating had taken place. The smear was also put on glass slides, dried after air drying, stained with Giemsa dye (Sigma-Aldrich, St. Louis, MO), inspected under a microscope, and photographed for the presence of sperm.
- The details of the mice who would become parents, including the date the vaginal plug was verified, were recorded and stored.
- Female mice were separated into two groups and housed in separate cages when the presence of a vaginal plug was established Group (1) is the control group, and group (II) is the treated group
- The treatment group got 2/4/6mg/kg body weight DEHP up to parturition

- Mice did not replace their cages until a few days before the due date (20 days after the vaginal plug was confirmed, removing any unnecessary excitement from the cage replacement). We closely adhered to the cage markings and provided information.
- During the mother's time nursing her young after delivery, cages were not changed the infant mice were weaned four weeks after delivery. The mice were then split into separate cages for the male and female mice to guarantee that each animal had enough room, with a maximum of five mice per cage.

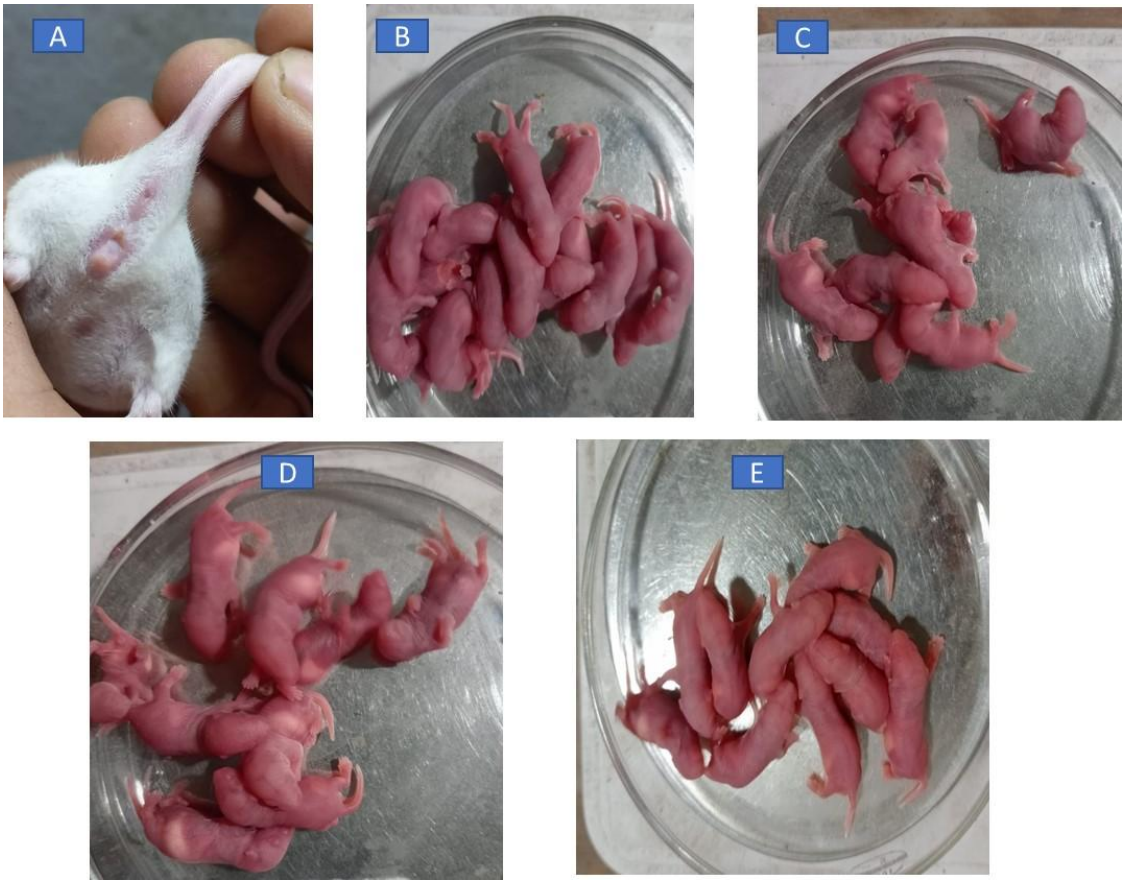

Photo 3.3: Breeding outcomes. A) Confirmation of plug in the female vagina B,C, D, E) Offspring from the experimental mice after parturition 2mg/kg DEHP, 4mg/kg DEHP, 6mg/kg DEHP respectively.

3.4. Observation of pregnancy outcome and post-natal body

The effect of DEHP on the post-natal growth of mice will be evaluated based on the growth of offspring from birth to weaning up to sexual maturity (8 weeks of age). For this purpose, E 0.5 mice will be treated with DEHP and continued up to the parturition period. After parturition, litter size (number of pups), litter weight, and weight/pups will be observed. Then pups will be observed until sexual maturity to see the body growth from birth to weaning up to sexual maturity.

3.4.1. Growth of offspring from birth to weaning

At birth, the gestational length, number of pups, average litter weight, number of dead pups, and number of pups with malformations were recorded (Weight of individual pups recorded from the litter size and weight of litter) The individual pups' weight (grams) was calculated by using the formula:

$$\text{Average weight of individual pups} = \frac{\text{Weight of litter}}{\text{Number of pups in litter}}$$

Following birth, pups were monitored daily to assess survival and development. Individual pups' weight (grams) was recorded separately (male, female) at 4 weeks of age to compare the body growth of control and DEHP-treated offspring and compare male and female growth rates. Body weight gain from birth to weaning was calculated using the formula

$$\text{Daily weight gain from birth to weaning} = \frac{\text{Final weight at 4 weeks of age} - \text{Initial weight at birth}}{\text{Day Number}}$$

3.4.2. Body growth of offspring from (weaning to sexual maturity)

After 28 days of weaning offspring were separated (male and female) from the cage and properly marked according to the number of offspring and kept in separate cages: The pups were monitored up to sexual maturity (8 weeks of age). They were supplied quality food with

balanced nutrition and provided water ad libitum. The body weight of each offspring was measured daily, from the 4th week to the 8th week of age (up to sexual maturity). Weight was measured by using an electric balance. Weight gain and the rate of weight gain for each offspring were calculated and compared between the control and DEHP-exposed offspring. For measuring weight, the cage containing offspring was placed on the table. Then placed a medium-sized glassbeaker on the analytical balance and properly noticed the scale of 0, removed an offspring from the cage and placed the offspring gently in the glass beaker, waiting until the mice remained stable, then the weight was recorded in the record sheet. This method was followed by each offspring for measuring weight. These data were calculated by using the following formula:

Body weight gain = Final body weight - Initial body weight

Rate of weight gain = Final body weight - Initial body weight Day

Here the body weight at 8 weeks of age was the final weight and the body weight at 4 weeks of age was the initial weight

Photo 3.4: Measuring weight of offspring. A) Measuring litter weight at birth B) Measuring weight at weaning to sexual maturity C) Recording body weight in daily record sheet.

3.5. Collection and preparation of samples

Once reached adulthood (after 8 weeks of age), adult mice of the F1 generation were started overnight and collected the following samples for experimental purposes.

3.6. Identification of estrous cycle in the offspring mice

Selecting mice that will mate when matched with a male to generate timely pregnancy depends on knowing the stage of estrous Proestrus, estrus, metestrus, and diestrus are the four stages of the mouse's estrous cycle, which lasts for four to five days. Evaluation of the estrous cycle in experimental animals proved helpful in this work for mouse breeding. In this study, we employed vaginal cytology to identify adults female mice's estrous cycles.

Table 3.1: Length of different phases estrous cycle of mice (Ajayi & Akhigbe, 2020)

Estrous pha	Cycle length (hours)
Proestrous	<24
Estrous	12-48
Metestrous	8-24
Diestrous	48-72

3.6.1. Steps for vaginal cytology (Ajayi & Akhigbe, 2020)

i. The mouse was first restrained (used the remaining 3 fingers of the non- dominant hand to apply pressure to the mouse's back and keep it largely still while holding the base of the tail firmly in place with the index and thumb.
ii. A cotton-tipped swab with an ambient temperature physiological saline solution was introduced into the vagina to collect a vaginal swab
iii. To remove the swab, it was softly rolled and rotated against the vaginal wall Rolling the cotton swab across the slide allowed cells to be transferred to a dry glass slide.
iv. After the slide had dried naturally, it was dyed for 45 seconds with around 400 mL of stain.
v. The slides were washed with water, covered with a coverslip, and then immediately examined under a microscope

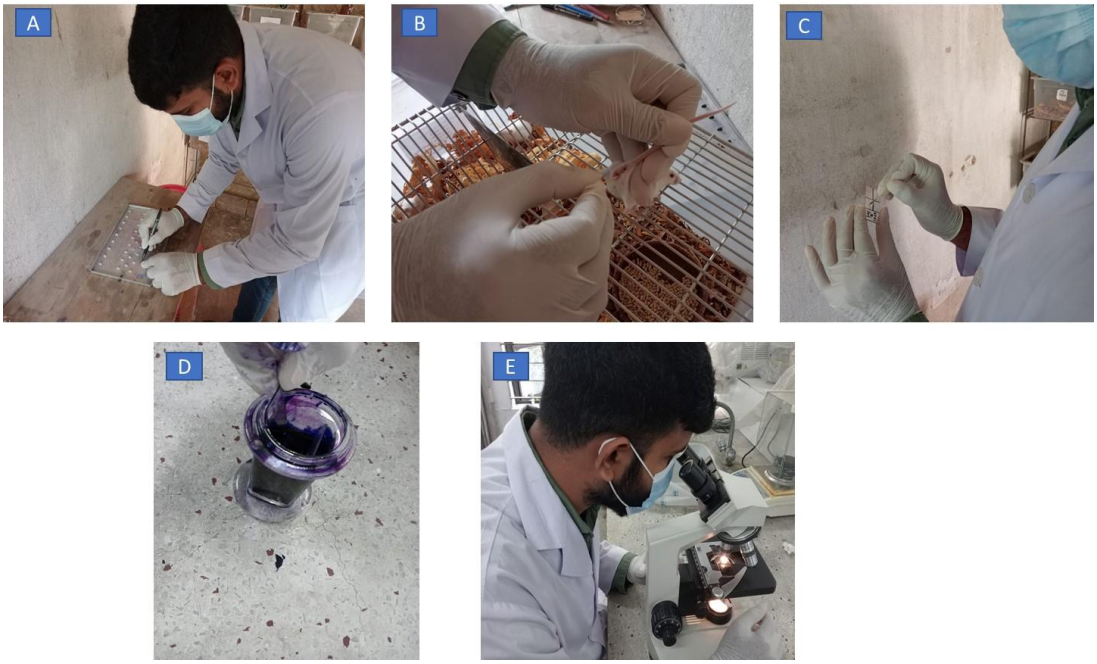

Photo 3.5: Sequential activities for vaginal cytology. A) Marking the glass slide B) Restraining and inserting the cotton tip into the vagina to collect vaginal swab C) Rolling the cotton swab on the glass slide D) Dying with Giemsa stain E) Observing the slide under a Microscope

3.6.2. Observation under a microscope

i. The estrous cycle stage was determined based on the presence or absence of leukocytes, comified epithelial cells, and nucleated epithelial cells.
ii. While the female is in proestrus, the bulk of the epithelial cells were nucleated, and some of them were comified Some leukocytes could be present if the female was in the early stages of proestrus
iii. As the cycle approached estrus, the cells were mostly comified epithelial cells .
iv. The presence of polymorphonuclear leukocytes and commified epithelial cells in vaginal swabs may indicate that the metestrus was a brief stage. Some nucleated epithelial cells were also seen in the late metestrus.
v. Diestrus, the longest stage, lasted for more than two days. With very few epithelial cells in late diestrus vaginal swabs, polymorphonuclear leukocytes predominated.

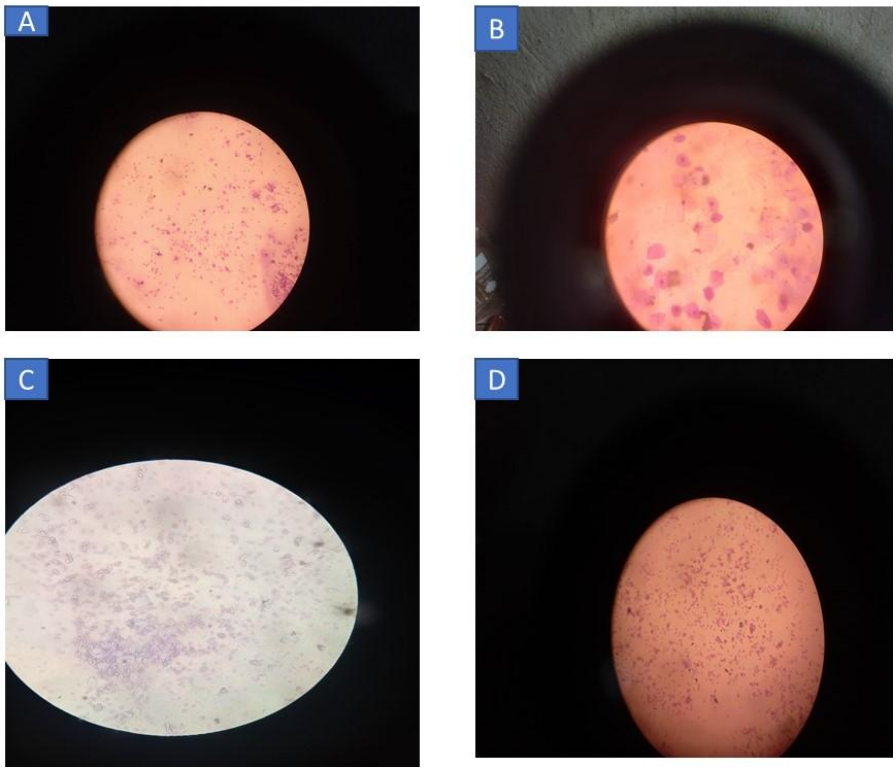

Photo 3.6: Microscopic observation. A) Cluster of epithelial cells B) Majority of cornified epithelial cells C) Some nucleated epithelial cells and leukocytes D) Mostly polymorphonuclear leukocytes

3.7. Collection of blood

Adult mice were deprived overnight and given mild diethyl ether anesthesia the next morning to draw blood. The animal was correctly recognized by the tail marking. Due to the need for a significant volume of blood, a cardiac puncture was used to collect the blood. The mouse was pinned to the experimental board's legs after being grasped by the scruff of skin just above the shoulders such that its head was up and its hind legs were down. Using a 1 ml syringe and a 22-gauge needle, we cautiously placed the needle into the mouse's heart after carefully locating the heart's exact location by exposing the mouse's interior cavity so that the heart was visible. To get the most blood possible, gently pull back on the plunger after blood started to emerge in the syringe. To prevent the heart from collapsing, blood was removed

gently. Blood was then preserved in a collecting tube for hematological and biochemical examination. The tube was correctly labeled with the date, mouse number, sample number, and sample name.

3.8. Collection of internal organs

After blood collection, the mouse was euthanized by cervical dislocation (Photo 3.8A) and Wetted the mouse with 70% ethyl alcohol then cut the abdominal skin by using a sharp scissor and forceps, Began the dissection starting from the initial incision below the navel going up toward the mouth To open the skin, a downward incision was made toward the tail, taking care not to cut through the membrane, uncovered the internal organs by carefully removing the abdominal membrane using scissors and forceps. Cut open the skin on the tape board side of the sternum, the bone at the front of the ribcage, Organs were identified for delicate separation and isolation using forceps and scissors, and each separated organ was stored individually in Petri dishes. Petri dishes were well cleaned and labeled with the number of animals, samples and collection date before organs were removed. By taking a clear picture of the assembled organs and visualizing the interior organs with any anomalies stored in separate Petri dishes for further examination, several organs including the liver, lungs, kidney, spleen, heart, uterus, and testicles were carefully removed.

3.9. Biochemical evaluation

Blood samples were immediately collected and transferred into clot activator tubes. A clot activator tube with silica particles coating the inner walls activated the coagulation process. Each sample was allowed to clot for a minimum of 30 min (maximum of 60 min). After clotting, the sample was centrifuged at 6000 rpm for 10 min. The serum or plasma then was pipette from the cellular elements (erythrocytes, platelets, leucocytes) and transferred to an acid-washed polypropylene tube, properly labeled, and stored at 4°C until ready for analysis.

Commercially available reagents were used according to the respective manufacturer's protocol for the measurement Follicular stimulating hormone and luteinizing hormone .All the parameters were compared between the control and exposed groups.

3.10. Evaluation of different organs

Organs were evaluated according to their morphology and relative organ weight (organ-to-body weight ratio).

3.10.1. Organ morphology

To observe the effect of DEHP, on organ development, organs namely the liver, lungs, spleen, heart, kidney, uterus, and testis were removed properly to evaluate based on gross morphology. After collection, the organs were washed with distilled water for removing blood. As a result, the organs were indisputably visualized to evaluate gross morphology. The criteria of gross morphological examination were based on the position, shape, size, color, and consistency of the organs. The organs were kept separately in different Petri dishes according to morphological features. Tissue paper was used to absorb water from the organs. Then the organs were placed on colored paper to take a clear photograph. Organ size was determined using a centimeter scale. According to the morphological feature, the organs were compared between the control group and the exposed group of offspring.

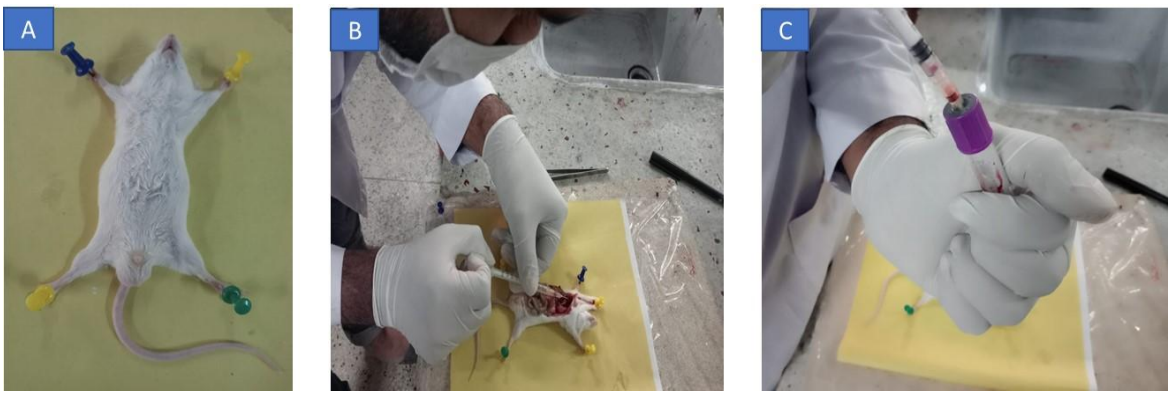

Photo 3.7: Collection of blood sample. A) Lying the mouse on the experimental board with anesthesia B) Collection of blood by cardiac puncture C) Storing blood in collection tube

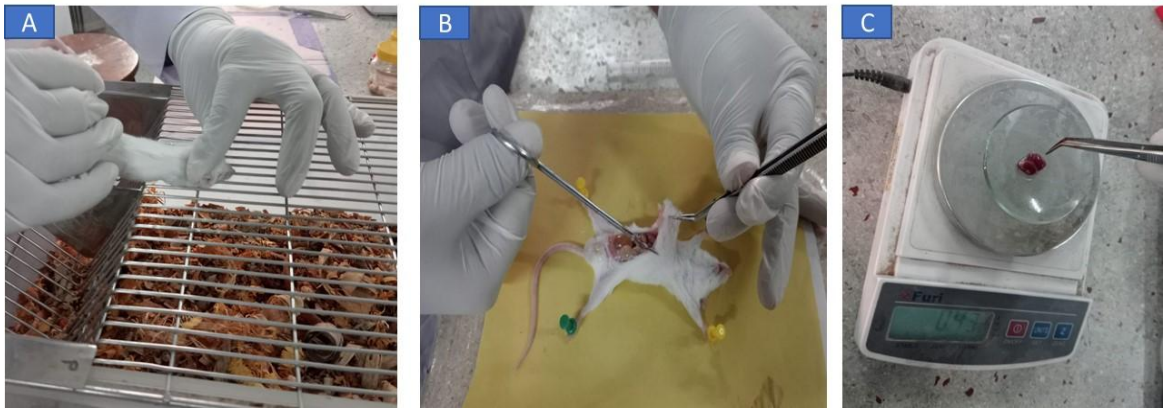

Photo 3.8: Collection of organs. A) Cervical dislocation of the mouse to euthanized B) Dissecting the mouse towards the mouth C) Measurement of organ weight

3.10.2. Organ developments

The body weight of the animal was taken before being euthanized after the removal of organs, all these organs were weighed individually using digital balance, and the organ-to-body weight ratio was calculated by dividing each animal organ weight by their body weight. This was called relative organ weight Organ to body weight ratio was calculated by the following formula:

Organ to body weight ratio= Organ weight Body weight × 100

Then the relative organ weights were then plotted, analyzed, and interpreted. The control and treated group organs were evaluated to compare the effect

3.11. Statistical analysis

Each experiment was repeated at least three times. The results of body growth, relative organ weight, and hematological and biochemical parameters were expressed as a ratio against each control and DEHP, exposed and are expressed as Mean ± Standard Error of Mean (SEM). Single-factor analysis of variance (ANOVA) was used to analyze the statistical differences between the control and exposed groups where significances were considered. Furthermore, the Student-Newman Keuls test will be used to compare the four groups.

CHAPTER 4

RESULTS

The present study evaluated the toxicity of DEHP on offspring body growth, estrous cyclicity, hematological and hormonal parameters, and organ morphology of offspring obtained from adult female mice exposed to DEHP from plug confirmation to parturition.

4.1. Effects of DEHP on litter weight and litter size

Maternal exposure of DEHP, the number of pulps per litter was recorded and DEHP exposures were decreased the litter size and their litter weight compared the control group**.**

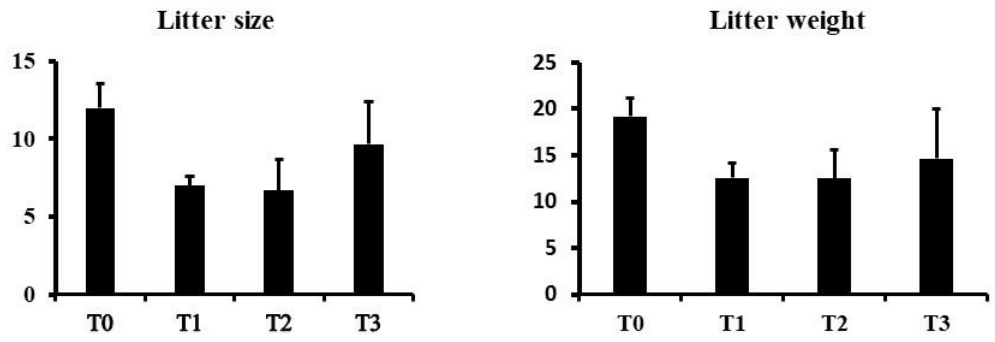

Fig 4.1: Effects of DEHP on litter weight and litter size

4.2. Effects of DEHP on postnatal growth of mice off spring

The study assesses the post netal (birth to weaning and weaning to sexual maturity) growth performance of offspring deprived from DEHP exposure in different groups and control group.

4.2.1 Impact of DEHP on body growth of F1 mice from birth to weaning

The result revealed that the T1 and T2 groups were increased the rate of body weight gain than the T0 group but T3 group was comparatively decreased the final body weight compared to T0 group.

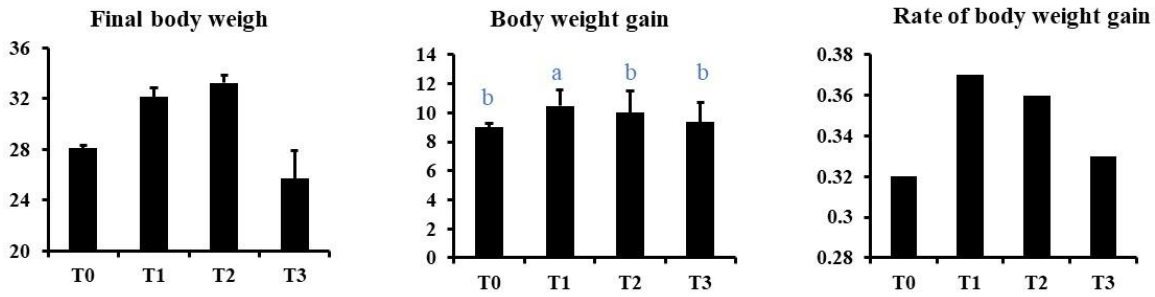

Figure 4.2: Impact of DEHP on body growth (from birth to weaning)

4.2.2 Impact of DEHP on body growth of F1 mice from weaning to sexual maturity

Phthalates parental exposure was inversely related with liter size in T1 and T2. In this study, the body weight of F1 mice (weaning to sexual maturity) was measured and compared between exposed and control group. The result revealed that the final body weight of T1 and T2 groups were significantly increased compared to T0 group.

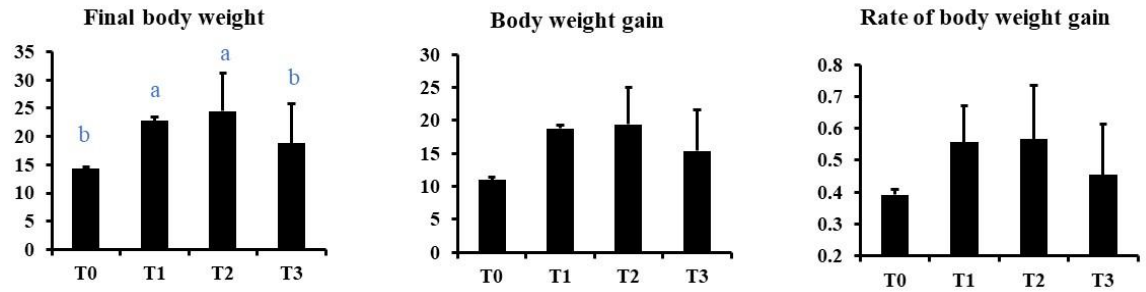

Figure 4.3: Impact of DEHP on body growth (from weaning to sexual maturity)

4.3. Impact of DEHP on estrous cyclicity

This estrous cyclicity study revealed that, the rate of estrous in the T1, T2, and T3 groups were gradually decreased compared to the T0 group, incase of proestrus T1 was increased compared to T0 group but T2, T3 group were decreased compared to T0 group. The rate of diestrus in the T1, T2 and T3 groups were increased compare to the T0 group.

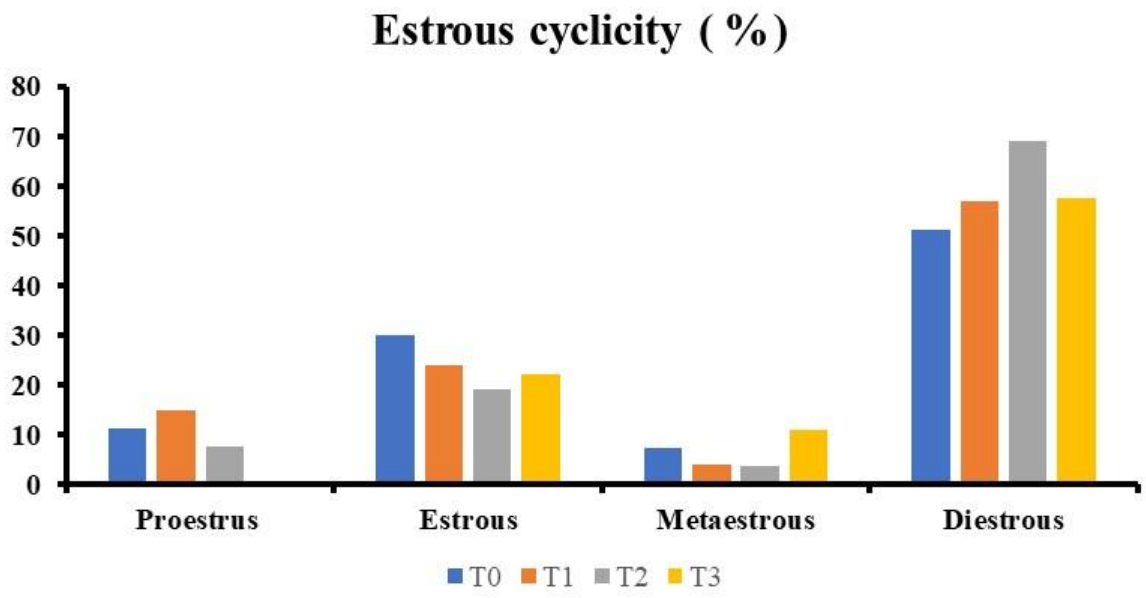

Fig 4.4: Impact of DEHP on estrous cyclist

4.4. Toxic effects of DEHP on organ development of mice offspring

In this study, the relative weight (% of body weight) of various organs (lung, liver, kidney, spleen, Heart, Thyroid, Uterus and ovary were measured and compared between exposed group and control group. The result showed that lung weight in the T1 and T3 groups were significantly increased from the T0 group but the T2 group was decreased from T0 group. In the T1, T2 and T3 groups, kidney and liver weight were significantly higher than in the T0 group. Heart weight in the T2 and T3 groups were significantly higher than in the T0 group. In the same way, spleen weight is comparatively higher in the T1 and T2 groups compared to the T0 group. There are no significant in thyroid, Uterus and Ovary.

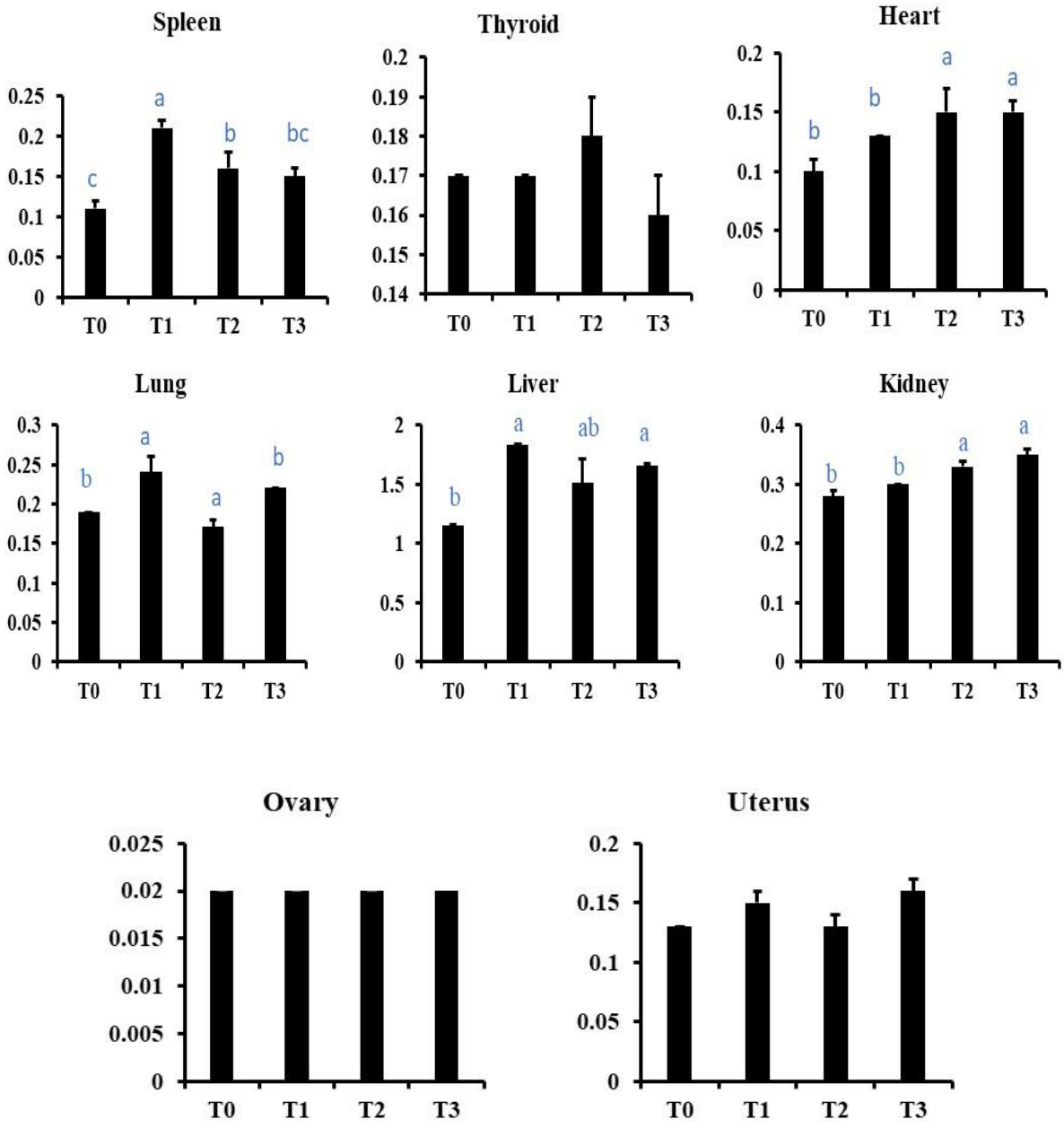

Fig 4.5: The organ to body weight ratio was assessed and compare between the control and DEHP exposed group of mice offspring. Values represent (mean ±SEM).

4.5. DEHP induced toxicity on organ morphology

The impact of DEHP on different organs was assessed after reaching the sexual maturity of F1 mice. Visual observation of the morphological characteristics of different organ showed that slight discoloration of liver and lung and substantially were affected in T3 treated offspring compared to T0. Interestingly, the size of the Liver in T1, T2 and T3 were comparatively larger than the group of T0. On the contrary, normal morphological feature of kidney, Spleen, Heart, Uterus and Thyroid was observed in control and different treatment groups.

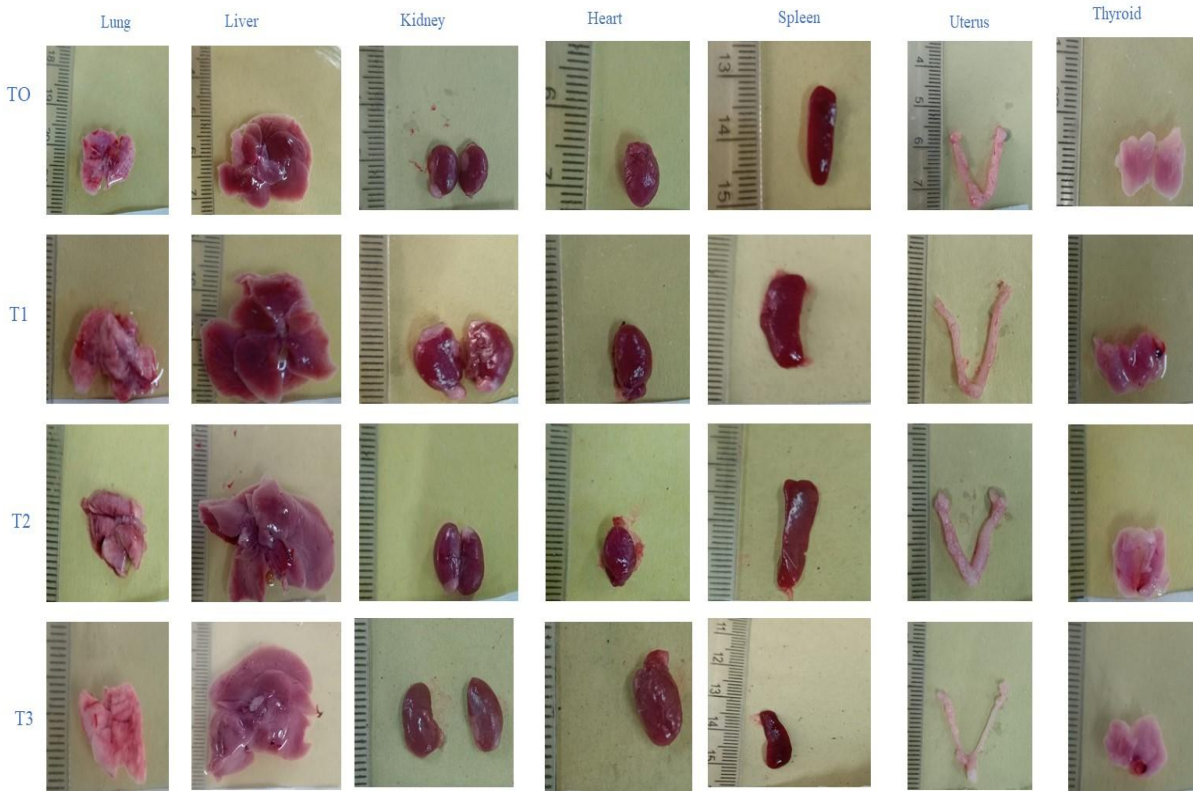

Photo 4.6: Effects of DEHP on different organs of F1 mice

4.6. Hematological alteration induced by DEHP exposure to mice off spring

The present study was aimed at measuring the changes in hematological parameters of blood tissue compared to the different phthalates exposed and control group of mice offspring. The result showed that the platelets in the T1, T2, and T3 groups were significantly higher than

T0 group. However, having a high platelet count can lead to blood clotting problems and, in some cases, may be associated with serious conditions like cancer. In the term of neutrophil percentage, the T3 group was significantly lower than the other groups, Lymphocyte percentage in the T1, T2, and T3 groups were significantly higher than the T0 group. High lymphocyte in blood levels were indicated that body was dealing with an infection or other inflammatory condition. In case of total WBC count in the T1, T2 and T3 groups were increased compared to T0 group. A high WBC count could indicate certain blood cancers or bone marrow disorders. The hematological parameters of PCT, RDW-CV, and RDW-SD were significantly lower in the T1, T2, and T3 groups than the T0 group. The percentage of monocytes in the T1, T2 and T3 were gradually decreased compared to T0 group. Monocytes were involved in the immune response to infection and low level monocytes counts were linked to increased rates of infection.

Table 4.1: Hematological parameter of control with different treatment group

Parameter	**T0**	**T1**	**T2**	**T3**
Haemoglobin (g/dl)	13.03±0.55	12.33±0.88	13.43±0.32	12.30±0.79
Total WBC count (/cmm)	3149.67±25.37	17413.33±10312.20	8650.00±199.75	4100.00±57.74
Neutrophil %	46.67±0.88a	46.00±0.58a	46.00±1.15a	42.00±1.15b
Monocytes %	3.00±0.58	2.33±0.33	2.00±0.58	1.67±0.33
Lymphocytes%	42.00±0.58b	52.67±0.67a	53.33±1.45a	55.00±0.58a
Neutrophil	4.06±0.06	3.98±0.01	4.02±0.54	3.57±0.73
Monocytes	0.63±0.03b	0.82±0.03a	0.17±0.01c	0.58±0.05b
Lymphocytes	3.58±0.46	4.67±0.63	4.25±0.37	4.29±0.18
Total Cir Eosinophil Count	431.67±25.69b	780.00±11.55a	0.33±0.33d	86.33±1.76c

Parameter	T0	T1	T2	T3
Platelet Count (/cmm)	104000.00± 577.35d	120000.00± 577.35c	190333.33± 2027.59b	281666.67± 2728.45a
MPV (fl)	5.90±0.59	6.26±0.63	5.23±1.18	8.93±1.82
PDW (%)	15.73±0.18	16.47±0.34	16.31±2.05	19.97±0.62
PCT (%)	9.74±1.06a	0.27±0.03b	0.10±0.00b	0.35±0.06b
PLCC (10^9/L)	79.00±4.62c	84.00±3.46c	126.00±1.73a	75.33±2.03b
PLCR (%)	4.70±0.06c	20.07±0.47a	6.80±0.13c	11.11±2.19b
RBC Count (Million/cmm)	6.32±0.32	5.64±0.46	7.16±0.54	5.41±0.36
HCT/ PCV (%)	37.97±1.82	36.74±0.83	32.07±5.00	32.58±1.28
MCV (fl)	46.00±2.31c	58.00±4.04b	46.33±0.88c	77.00±1.73a
MCH (pg)	17.00±0.58c	21.00±1.15ab	18.33±0.88bc	23.10±0.59a
MCHC (g/dl)	38.33±3.18b	50.00±4.04a	39.00±2.89b	34.67±1.76b
RDW-CV (%)	37.39±0.97a	18.02±0.19b	33.37±6.26a	15.30±0.65b
RDW-SD (fl)	73.86±1.51a	45.43±1.77c	61.49±2.75b	41.33±5.78c

The hematological parameters of mice are assessed and compared between exposed and nonexposed mice. The value represents (mean ± SEM TC: total count; WBC: White blood cell; Hb: Hemoglobin; RBC: Red blood cell; PVC: Packed cell volume; MCV: Mean capsular volume; MCH: Mean capsular hemoglobin; MCHC: Mean capsular hemoglobin concentration; RDW-SD: Red blood cell dimension width standard deviation; RDW-CV: Red cell distribution width-coefficient of variation.

4.7. Hormonal alteration induced by DEHP

This study also revealed that the serum biochemical alteration in DEHP-exposed group and compared to control group. The result showed that the value of Estrogen in T1, T2 and T3 groups were gradually decreased than T0 group. Consequently, progesterone in T3 group significantly lower than T0 group.

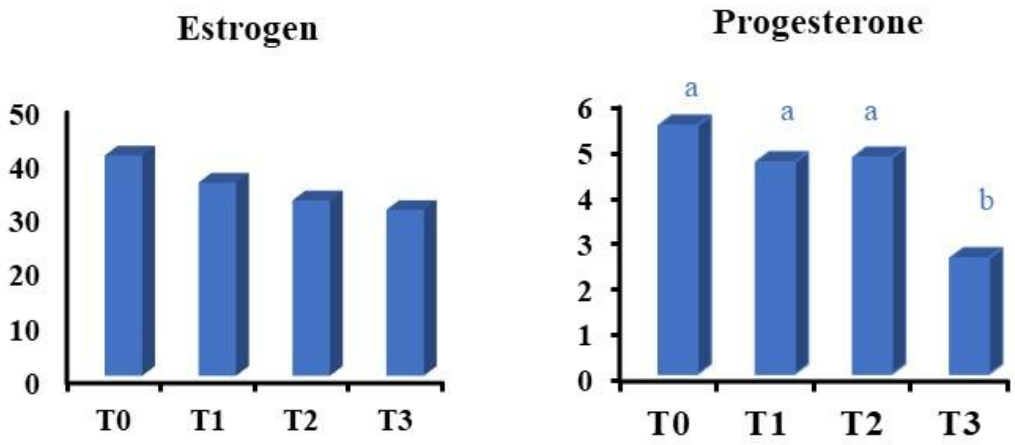

Figure 4.7 Hormonal alteration induced by DEHP

CHAPTER 5

DISCUSSION

DEHP crosses the placental barrier fast, which eventually has an impact on the development of the fetus and the offspring's later years. Some research is worried about how human pregnancy may be affected by ingesting or breathing in DEHP. In this work, following plug confirmation until parturition (19 days), we examined the effects of phthalates-induced toxicity on body development, as well as the hematological, hormonal, and organ morphological changes in the offspring. The current study demonstrates that the offspring's body weight throughout weaning to sexual maturity, the gestation time, litter weights, and litter sizes have no impact for this group. Compared to the control groups, the 2mg/kg DEHP groups' body weight considerably reduced over the observation period from parturition to weaning. Thyroid hormone is necessary for developing hormones, metabolism, and brain growth. It is believed that chemical exposure causes disturbance of the endocrine system in adults, children, and the developing embryo. Epidemiological study indicates that exposure to environmental phthalates may disrupt thyroid function and affect the development and growth of the body. Development hormones are thought to be indicators of longitudinal somatic development and are regulated by the thyroid hormone (Ohlsson, 1998). This outcome is in line with past research findings reported by Aydemir *et al*. (2018). Dong *et al*. (2019); Ran *et al*. (2019); and Parra Forero *et al*. (2019). It has been demonstrated that hematological and biochemical parameters are trustworthy markers for assessing the state of health in both people and animals (Saxena *et al*., 2011). Hemopoiesis is negatively disrupted by toxic chemicals, which also affect mature blood cell activity and metabolism. As a result, blood cell production may be aberrant, decreased, or compromised. According to Blaxhall *et al*. (1972); Duthie *et al*. (1985), hematopathology is employed as a health status indicator in a variety of species to identify physiological changes brought on by diverse stressors, such as

exposure to pollution, illnesses, metals, hypoxia, etc. WBC, RBC, Platelets, RDW-SD, RDW-CV, MCV, MCH, and MCHC were all observed throughout this examination. The white blood cell count indicates how many white blood cells are present in the blood. White blood cells make up the immune system. They help your body fight against infections and ailments. One sign of the continuous inflammatory reaction in the body is the white blood cell (WBC) count in peripherally circulated blood. One kind of phagocyte that is typically seen in the circulation is the neutrophil. Neutrophils are among the first inflammatory cells to move toward the site of inflammation during the early (acute) phase of inflammation, which is often brought on by bacterial infection, exposure to the environment, and some types of cancer (Jacobs *et al*., 2010). By following chemical cues like interleukin-8 (IL-8), C5a, fMLP, leukotriene B4, and hydrogen peroxide (H2O2), they move across blood arteries and subsequently into interstitial space. This process is known as chemotaxis. They make up the majority of the cells in pus, which explains why it appears pale or yellowish. Yoo (2011). T3 group considerably lower than control group in this study; this is comparable to Wang *et al*., 2020; Yirong *et al*., 2020; Vetrano *et al*., 2010. Current research has shown that DEHP inhibits apoptosis, with a larger impact in neonates. Imply that newborns' exposure to phthalates may worsen their neutrophil clearance, aggravating their inflammatory disorders. In reaction to chemokines produced at the sites of infection or damage, neutrophils gather in tissues. Strong neutrophil chemoattractant IL-8 also increases the expression of molecules involved in cell adhesion (Scapini *et al*., 2000). The current investigations show that, in adult cells, MEHP increased fMLP-induced chemotaxis, but not in neonatal cells. This was associated with mature neutrophils producing more IL-8. Additionally, it has been demonstrated that MEHP increases the expression of the integrin CD11b and induces calcium flow, both of which are critical for cell motility (Niggli *et al*., 2003). Neonatal neutrophils have been shown to have developmental abnormalities in terms of calcium mobilization and

CD11b expression (Agarwal *et al*., 19986). These flaws might be a factor in MEHP's suppressive effects on chemotactic responses in these cells, as could the fact that MEHP cannot induce IL-8 synthesis in neonatal cells. The current research demonstrates that adult and neonatal neutrophils produce considerable amounts of H_2O_2. On the other hand, newborn cells produced more H_2O_2 overall and at a faster pace, which is in line with our earlier findings (Weinberger *et al*., 2007). Additionally, we discovered that MEHP, although only in newborn cells, increased the expression of NOX1. According to Lambeth *et al*. (2007), NOX1 is the primary enzymatic mediator of neutrophil superoxide anion production. The elevated expression of NOX1 by neonatal neutrophils aligns with earlier findings that these cells generate more superoxide anion in response to inflammatory stimuli than do adult cells (Tsukimori *et al*., 2007). Our finding that constitutive expression of NOX1 was lower in newborn cells than in adult cells is noteworthy. These results corroborate observations that these cells have negligible or no constitutive generation of superoxide anion (Newburger *et al*., 1982). The notion that these antioxidants are essential for shielding neonates from reactive oxygen species produced in the fetal and maternal circulation is supported by the interesting finding that constitutive expression of SOD and catalase was higher in neonatal neutrophils compared to adult cells (Rosenfeld *et al*., 1986). MEHP showed no effect on SOD or catalase expression, in contrast to its stimulatory effects on NOX1 and oxidative metabolism in neonatal neutrophils. Reactive oxygen species and NOX1 production are elevated by MEHP without corresponding increases in antioxidant production. Further comparison analyses demonstrated a considerable reduction in the constitutive production of RANTES, VEGF, MIP-1β, IL-6, IL-8, IL-1β, and IL-8 in newborn neutrophils relative to adult cells. These results support earlier observations that newborn cells have developmental defects in the production of chemokines and cytokines (Carr *et al*., 2000). While MEHP inhibited MIP-1β production in mature neutrophils, it inhibited RANTES generation in

newborn cells. CC chemokines MIP-1β and RANTES mainly target macrophages and monocytes (Baggiolini *et al*., 1995). The fact that MEHP downregulates these mediators indicates that neutrophils are the primary target of MEHP's pro-inflammatory effects. Eosinophils, often referred to as acidophils or, less frequently, eosinophils, are a subset of white blood cells that are part of the immune system that fights infections and multicellular parasites in vertebrates. They also regulate basophils and mast cells as well as the systems linked to asthma and allergies. These are granulocytes that grow in the bone marrow during hematopoiesis and then migrate into the blood, where they undergo terminal differentiation and cease to proliferate. They make up between 2 and 3% of the body's white blood cells. DEHP oxidation results in inflammation, which lowers eosinophil levels. Demonstration of potentiated lymphocyte-dependent production of the T2 cytokine IL-4 by DEHP administered i.p. (Lee *et al*., 2004) and IL-4 and IL-10 following long-term exposure to inhaled aerosols with DEHP (Larsen *et al*., 2007) suggested an adjuvant effect on IgE and IgG1. After prolonged inhalation of aerosols, MEHP increased the release of IL-4, IL-5, and IL10 (Hansen *et al*., 2007). Remarkably, DEHP and MEHP, respectively, enhanced IFN-c secretion in that study and two others (Lee *et al*., 2004; Larsen *et al*., 2007), indicating an impact on Th1 cells. No adjuvant effect on IgG2a was reported in the same papers although by Larsen & Nielsen (2007) after i.p. administration of DEHP. Other term platelets are small, colorless cell fragments in our blood that form clots and stop or prevent bleeding. Platelets are made in our bone marrow, the sponge-like tissue inside our bones. Red blood cells, white blood cells, and platelets can all be produced from stem cells found in bone marrow. The study's findings showed that the DEHP-exposed group's platelet count increased significantly in comparison to the control groups. The information provided by Aydemir *et al*. (2023) and Yalçin *et al*. (2022) supports this conclusion. This study suggests that increased platelets or thrombocytosis could indicate a sudden sort of hypersensitivity to DEHP exposure. When

determining how much of a health risk exposure to environmental contaminants poses, platelet parameters and inflammatory cell numbers are essential tools. In systemic lupus erythematosus, for instance, mean platelet volume (MPV) is a marker of the disease activity and the local inflammatory response (Delgado-García *et al*., 2016). Hepatic inflammation is measured using platelet count (PLT) and platelet distribution width (PDW), both of which are considered valid indicators (Pan *et al*., 2016). This study suggests that higher platelet counts, or thrombocytosis, could indicate immediate-type hypersensitivity to DEHP exposure. These results are consistent with those of Selamoglu Talas *et al*. (2012) who found that the kids of the DEHP-exposed group had considerably greater levels of MCV, MCH, and MCHC than did the offspring of the control group. Macrocytic type anemia could be the reason for the elevated MCV, MCH, and MCHC values. The examination of serum biochemical parameters is advised to provide early warming signs of substantial modifications in stressed animals, according to Kumar *et al*. (2016). This is especially helpful for determining the target organs of toxicity as well as the overall health status of animals. To further illustrate the toxicity of different tissue systems, biochemical studies were used. DEHP has the potential to have toxicological effects and biochemical disturbances, both of which are serious health dangers, according to Messiah *et al*. (2013). It has been determined that the red blood cell distribution width (RDW), a property provided by total blood cell count assays, is an inflammatory biomarker. One of the metrics 57 commonly reported in the whole blood cell count test, the red blood cell distribution width (RDW) indicates the variability in mature erythrocyte size in peripheral blood and the inefficiency of bone marrow erythropoiesis (Simel *et al*., 1988). For many years, it has been utilized in routine practice to establish a differential diagnosis for various forms of anemia, such as iron deficiency anemia (Van Zeben *et al*., 1990). Recent research has revealed that RDW is an inflammatory biomarker in several conditions, such as chronic lung diseases, acute and chronic kidney diseases, cardiovascular diseases (Tonelli *et*

al., 2005; Arbel *et al*., 2014), and critically ill patients (Wang *et al*., 2012; Purtle *et al*., 2014). In this study, the RDW-CV and RDW-SD in the DEHP groups are significantly lower than in the control group. These results differ from those of papers by Ofem *et al*. (2014) and Karzan *et al*. (2019). Estrogen and progesterone in this study are lower than normal, which is consistent with findings from Saadeldin *et al*. (2018) and Somasundaram *et al*. (2016). The development and maturation of female reproductive organs as well as the onset of regular menstrual cycles are all impacted by the ovarian sex steroid hormones, progesterone and estrogen. The precursor of steroid hormones is cholesterol that is synthesized de novo from acetate or that is taken in as LDL-cholesterol via LDL-receptor. In primary cultures of rat granulosa cells, the effects of MEHP on steroid hormone synthesis were investigated in order to identify the molecular mechanism by which DEHP/MEHP decreased estradiol in the granulosa cell. Because granulosa cells differentiate and produce steroidogenic enzymes in response to follicle-stimulating hormone (FSH), primary cultures of these cells are a suitable system for studying hormone synthesis in vitro (Erickson 1983). In primary culture, the hormonal effects of FSH are very similar to normal metabolic processes in vivo (Erickson 1983). The pituitary hormones FSH and LH, which activate granulosa and thecal cells, respectively, regulate the generation of ovarian hormones in vivo (Liu and Hsueh 1986). The gonadotropins LH and FSH bind to certain G-protein-coupled receptor sites, which activates adenylate cyclase and causes the build-up of cAMP, which activates enzymes involved in steroidogenesis (Hsueh *et al*. 1984). Both granulosa and thecal cells use the cholesterol side-chain cleavage enzyme (P450scc) to convert cholesterol to progesterone. P450scc is triggered by intramitochondrial transfer of cholesterol to the enzyme and by FSH-induced cAMP, which activates the enzyme (Hsueh *et al*. 1984). The primary steroidogenic intermediate shared by all steroid hormone classes is prostenolone. The enzyme 3β-hydroxysteroid dehydrogenase-isomerase converts it to progesterone. MEHP did not function as an enzyme

inhibitor; rather, it reduced the maximal activity of aromatase (Davis *et al*. 1994b). As a result, MEHP changed either the amount of synthesis or the rate at which the enzyme was degraded in the granulosa cell, affecting the availability or levels of aromatase. Although MEHP activates PPAR receptors (Corton *et al*. 2000), there is no evidence that MEHP directly binds to PPAR. Thus, activation of this receptor by MEHP occurs either through release of endogenous fatty acids from FABPs or through a yet unidentified intermediate factor. Activation of both PPARα and PPARγ by MEHP in the granulosa cell results in decreased transcription of aromatase, the primary mechanism of its female reproductive toxicity. Activation of both PPARα and PPARγ also increases mRNA for FABP in the granulosa cell (Lovekamp-Swan *et al*.200), creating more binding proteins for PPAR activators. Decreased estradiol synthesis and increased estradiol metabolism contribute to suppressed estradiol levels after MEHP treatment. It is possible that the phthalate may also affect other steroid or peptide signaling pathways, causing effects on estrous cyclicity. Circulating progesterone levels are highest in diestrus/metestrus and lowest in estrus (Walmer *et al*. 1992). Progesterone levels are required for the maintenance of pregnancy (Milligan and Finn 1997). Endocrine-disrupting chemicals are known to cause nonmonotonic dose responses, specifically for receptor-mediated effects (Vandenberg 2014). Steroid hormones, such as estrogens and progestins, and peptides, such as inhibits and activins, regulate the estrous cycle via the concerted action of positive and negative feedback loops on the hypothalamus-pituitary-ovarian (HPG) axis. The current findings together with previous results indicate that the phthalate mixture affects estrogen signaling in a nonlinear manner to affect estrous cyclicity. Females exposed to phthalates showed a substantial increase in liver weight when compared to the control group. Studies have shown a considerable rise in liver weight that is consistent with the findings of Rusyn *et al*., (2006), Aydemir *et al*., (2018), Radha *et al*., (2020), and Batool *et al*., (2022). An increase in the proliferation of hepatic

peroxisomes is the most prominent cellular effect of phthalate treatment in rats. Pugh *et al*. (2000) state that there is a possibility that this alteration and the inhibition of GJIC contribute to the development of liver cancer in mice. Rat exposure to phthalates appears to be the cause of both hyperplasia (an increase in the number of cells) and hypertrophy (an increase in the size of each cell) of liver parenchymal cells. Peroxisome proliferators cause an increase in the activity of the enzymes that oxidize DNA, lipids, and other substances in addition to producing and degrading hydrogen peroxide. It was initially proposed by Reddy and colleagues (Rusyn *et al*., 2012) that the peroxisome's fatty acyl-CoA oxidase was the enzyme responsible for oxidative stress. Based on a review of numerous highly referenced and varied reviews (Caldwell 2012; Guyton *et al*., 2009; Klaunig *et al*., Roberts *et al.* 2007; Rusyn and Corton 2012, Kushman *et al*., 2013) identified nine mechanistic events for DEHP and its metabolites in the liver. 52 The major cellular processes are as follows: (1) PPAR (peroxisome proliferation activation receptor) activation (2) peroxisome proliferation; (3) cell proliferation; (4) activation of other nuclear receptors; (5) activation of Kupffer cells; (6) suppression of hepatocellular apoptosis; (7) oxidative stress; (8) inhibition of gap-junctional intracellular communication (GJIC); and (9) genotoxicity. PPAR participates in fatty acid absorption and transport, ketogenesis, and lipogenesis in the liver. The hallmarks of PPAR activation include (1) an increase in peroxisome size and number (i.e., peroxisome proliferation); (2) an increase in acyl Co-A oxidase or CYP4A gene expression, protein level, or activity (i.e., lauric acid hydroxylase); and (3) an increase in carnitine acyl Co-A transferase levels. Additionally, PPAR is in charge of the surge in hepatocyte proliferation that is observed in rats in response to peroxisome proliferating substances such as DEHP. The generation of ROS (a marker of oxidative stress) increases as a result of PPAR-mediated induction of peroxisomal and microsomal enzymes in the liver of rodents (Kushman *et al*., 2013). To investigate the underlying mechanisms of the potential BPA cardiotoxicity,

AboulEzz *et al*. administered BPA orally to adult male rats for 6 to 10 weeks (25 or 10 mg/kg/day) and examined oxidative stress parameters in the heart. They discovered lower levels of reduced glutathione (GSH), increased lipid peroxidation, and decreased catalase activity, suggesting that exposure to BPA hampered the function of mitochondria and produced reactive oxygen species (ROS) in the hearts of male rats. Nitric oxide (NO), which may cause vasoconstriction and a reduction in the heart's blood supply, was also lowered as a result of BPA exposure. Lower acetylcholinesterase activity in the BPA-treated group may have resulted in decreased ventricular contraction and heart rate. These findings revealed that heightened oxidative stress can contribute to the possible negative effects of phthalate the cardiovascular system. The kidney is a vital organ that eliminates waste and surplus fluid from the body. It also eliminates acid created by human cells and keeps the body's levels of salt, water, and minerals like potassium, calcium, and sodium in a healthy range. Nerves, muscles, and other tissues might not function correctly without this equilibrium. Additionally, it is a 53-year-old male hormone that supports healthy blood cell formation, blood pressure regulation, and robust, strong bones. The blood's elevated BPA levels may be an indicator of kidney damage. From a functional perspective, it seems sense that the kidney's decreased ability to filter substances would result in an increase in the concentration of that compound in the blood. It is reasonable to conclude that this rise results from the kidney's incapacity to carry out its function naturally if we simply consider the research of Krieter *et al*. (2013) and Shen *et al*. (2019). Nonetheless, the two longitudinal investigations by Hu *et al*. (2016) examine their research group across time, lending more support to the raised hypothesis. Regarding lung toxicity, per internal administration of DEHP to experimental animals has produced conflicting results (FDA/CDRH, 2001). Sjöberg *et al*. (1985) gave 40-day-old rats six infusions of a 500 mg/kg bw DEHP emulsion over the course of 12 days. The emulsion had a mean particle diameter of less than 0.8 μm with "occasional particles" between 2 and 5

μm found no lung lesions. Although no granulomas were found in the current study, the size of the lipid emulsion's particles increased as the dose of DEHP increased (Supplemental Figure 4). It is possible that these larger lipid globules contributed to the granulomas' presence, as the incidence or severity of granulomas in PND 23 animals did not reflect the magnitude of the increase in large particles with increasing dose (Driscoll, 2009; Driscoll *et al*., 2006). This is because large particles can accumulate in lung and other organ capillaries. The increase in the kidney and liver weight to body weight ratios found in this study, as well as earlier investigations, may have been caused by this particle size impact (Nordstrand *et al*., 1987). Adult rats injected with emulsions of the plasticizer DINCH in 20% Intralipid for 28 days also showed microgranulomas in the livers, spleens, and lungs (David *et al*., 2015). To determine whether DEHP or other plasticizer emulsions contribute to the formation of granulomas or other lung lesions, care would need to be taken in synthesizing these emulsions to obtain a particle size distribution similar to the 20% Intralipid vehicle control. According to several studies, the offspring of dams treated orally with high doses of DEHP (> 1,000 mg/kg bw/day) during late gestation and lactation (Rosicarelli and Stefanini, 2009) or with a high dose (750 mg/kg bw/day) given intraperitoneally to neonates (Liang *et al*., 2018) experienced a delay in lung development with decreased formation of alveolar septa. We observed a similar effect in the 600 mg DEHP/kg bw/day group of both exposure routes. This effect is like that observed in the bronchopulmonary dysplasia that is common in premature infants and that has complex causes, including exposure to high oxygen concentrations and mechanical respiration (Bancalari and Jain, 2019; Kalikkot Thekkeveedu *et al*., 2017). Others (Liang *et al*., 2018; Magliozzi *et al*., 2003; Rosicarelli and Stefanini, 2009) have suggested that DEHP may worsen this condition in the NICU. Although this effect was only convincing at the highest DEHP dose in this study, lower dose effects in the IV arm cannot be ruled out. Similar to the induction of granulomas, this problem of lower dose effects might only be

remedied by using DEHP preparations whose particle size distribution is comparable to that of the Intralipid vehicle and by performing morphometric analyses on lungs that are consistently inflated at necropsy. The spleens of animals and fowl are essential immunological organs. Effects of immunosuppression on the development of immune organs and the proportional mass of immune organs, according to Fan *et al*. (2013), is a good indicator of an organism's development and immunological function. The finding from this study is that spleen weight is significantly increased compared to the control group. This outcome is similar to previous findings observed by Cheng *et al*., (2017); J. Kelleher., (1990).

Visual observation of morphological characteristics of different organs showed slight discoloration of the liver, which was subsequently affected in the phthalates-treated mouse compared to the control group and different treatment groups. Interestingly, the size of the liver in the phthalates group was comparatively larger than in other groups. On the contrary, normal morphological features of the kidney, lung, liver, spleen, heart, uterus, and urinary bladder were observed in control, 2mg/kg DEHP, 4mg/kg DEHP and 6mg/kg DEHP treated adult female mouse. That is similar to Zhang *et al*., 2017. David *et al*., 2000.

CHAPTER 6

SUMMARY AND COCLUSIONS

The present study was designed for evaluation of the Phthalates-induced toxicity on body growth and assessed hematological and hormonal alteration and organ morphology of offspring after DEHP exposure during plug confrontation day to parturition in adult female mice at the Laboratory Animal Facilities of the Department of Genetics and Animal Breeding, of Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh. Swiss albino mice of either sex, 6-8 weeks of age, and weighing 25-30 g was obtained from the Animal Resources Facility (ARF) of the International Centre for Diarrheal Disease Research, Bangladesh (icddr, b) Five mice per cage for female mice and one mouse per cage for male mice were selected randomly and housed in plastic cages with wood-cube bedding They were kept in an animal house with plenty of ventilation. The housing was kept at a temperature of 26±2°C with a relative humidity of 30-70% and provided the animals with complete access to food and water and housed under-regulated lighting (12-hour light/dark cycle). They were provided regular food in the form of pellets. The amount of water consumed was recorded before the beginning of the research, the mice were maintained for a week to acclimate to the new surroundings, and the cages were marked with the appropriate introduction. The study was carried out with different litter-weaned male (n=12) and female (n=12) mice. Male and female mice were randomly selected and mated with male mice from separate cage. The mating strategy was two females and one male. The female was examined each morning for the presence of semen plug. The detection of semen plug was designated as embryonic day 0.5 (E0.5) of gestation. Plug positive females were considered "pregnant" and immediately placed in separate cage for treatment. Pregnant female mice were randomly divided into experimental group, namely T0 (control), T1 (2mg/kg DEHP), T2 (4mg/kg DEHP) and T3 (6mg/kg DEHP) groups. During the 0.5 days of gestation, different amount of

phthalates was supplied that are commercially available DEHP, sigma Aldrich co. That is mixed with same amount of DMSO and distilled water and the control group received filtered normal water up to lactation period. Drinking water changed every other day to prevent Oxidation. The volume of water was same for both the control and treated group throughout the experiments. At birth, the number of puppies, average litter weight and the number of pups with malformations were recorded. The individual pulps weights (gm) was calculated by using the formula: average weight of individual pulps= weight of litter/number of pulps in the litter. Following birth, pups were monitored daily to assess their survive and development. At 4 weeks of age, individual pulps weights (gram) were recorded separately (male and female) and separated (male and female) in new cage. Properly marked according to the number of offspring and kept in separate cages. Body weight gain from birth to weaning was calculated using the formula. (Final weight at 4th weeks of age -initial weight at birth)/number of days. The body weight of each offspring was measured daily, from the 4th weeks to 8th weeks (Upto sexual maturity) by using electric balance. Body weight gain and rate of body weight gain of mice offspring were recorded. After 8 weeks Evaluation of the estrous cycle in experimental animals proved helpful in this work on mouse breeding. In this study, we employed vaginal cytology to identify the estrous cycles of adult female mice Proestrus, estrus, metestrus, and diestrus are the four stages of the mouse's estrous cycle, which lasts for four to five days. We recorded 5 cycle in every groups mouse and were recorded. Adult mice were deprived overnight and given mild diethyl ether anesthesia the next morning to draw blood. The mouse was pinned to the experimental board's legs after being grasped by the scruff of skin just above the shoulders such that its head was up, and its hind legs were down. Blood was collected directly from the cardiac puncture by using a 1 ml syringe and transferred into clot activator tubes and lithium heparin zed tubes for biochemical analysis as well as hematological analysis, or a complete blood count complete blood count

(CBC) for analyzing the RBC, WBC, differential blood count (neutrophils, eosinophils, basophils, monocytes, and lymphocytes), Hb, PLT, PCV, ESR, TCE, MCV, MCHC, MCH, RDW-SD, and RDW-CV by using an automated hematology analyzer (Mindray, BC-20) For biochemical analysis, the blood sample was centrifuged at 6000 rpm for 10 min for the separation of serurn. Then the serum was taken by pipette and mixed with available reagents (CHRONOLAB, RANDOX TRIGS-RIB, RANDOX UA-RIb) for analyzing Estrogen and Progesterone by using a biochemical analyzer (Humalyzer Primus) Cut open the skin on the tape board side of the sternum, the bone at the front of the cage, and organs, namely the liver, heart, lung, kidney, spleen, testis, and uterus, were identified for delicate separation and isolation using forceps and scissors, and each separated organ was stored individually in Petri dishes. Organs were evaluated according to their morphology and relative organ weight (organ-to-body weight ratio). The criteria of gross morphological examination were based on the position, shape, size, color, and consistency of the organs by visual examination. Organ size was determined using a centimeter scale. The organs were kept separately in different Petri dishes according to morphological features. Organs were weighed individually. Using a digital balance and the organ-to-body weight ratio was calculated by dividing each animal's organ weight by its body weight and multiplying it by a hundred. Then the organ placed on colored paper to take a clear photograph. Finally all the data on body growth, relative organ weight, hematological and hormonal parameters were collected and expressed as a ratio against each control and DEHP was exposed as Mean +-, standard error of mean (SEM). Single factor analysis of variance (ANOVA) was used to analyze the statistical difference between control and treatment groups where significantly considered at $p<0.005$. Furthermore, the student-Newman-Keuls test will be used to compare the four groups. Difference will be considered significant at the level of $p<0.005$.

The study assess the post netal (birth to weaning and weaning to sexual maturity) growth performance of offspring deprived from DEHP exposure in different groups and control group.

The result revealed that the offspring of 2mg/DEHP, 4mg/kg DEHP and 6mg/kg DEHP slower increase rate of body weight gain than the offspring of control mice that were not given any treatment. Phthalates parental exposure is inversely related with liter size in 2mg/kg DEHP and 4mg/kg DEHP. In this study, the body weight of F1 mice (weaning to sexual maturity) was measured and compared between exposed and control group. The result revealed that the final body weight of offspring 6mg/kg DEHP groups is significantly decrease than normal value. Estrous and diestrus show 2mg/kg DEHP groups mice compared to the control group.

the relative weight (% of body weight) of various organs (lung, liver, kidney, spleen, Heart, Thyroid and Uterus and the organ to body weight ratio were measured and compared between exposed group and control group of F1 mice. The result showed that lung and liver weight in T0 and T3 groups significantly increase than standard value. In case of T2 and T3 groups kidney weight is significantly increase than control group. Heart weight in T2 and T3 groups is significantly increase than control group. In the same way spleen weight is comparatively higher in T1 and T2 groups compared to the T0 group. There are no significant in thyroid, Uterus and Ovary.

The impact of DEHP on different organs was assessed after reaching the sexual maturity of F1 mice. Visual observation of the morphological characteristics of different organ showed that slight discoloration of liver and lung and substantially were affected in T3 treated offspring compared to bon treated offspring. Interestingly, the size of the Liver was comparatively larger than the group of control offspring. On the contrary, normal morphological feature of kidney, Spleen, Heart heart, Uterus and Thyroid was observed in

control and different treatment group. This study was aimed at measuring the changes in hematological parameters of blood tissue compared the different phthalates exposed and control group of mice offspring. The result showed that PCT, RDW -CV and RDW-SD were significantly decrease in T1, T2 and T3 groups than T0 group. Whereas platelets in T1, T2 and T3 groups significantly higher than T0 group. In case of neutrophil % in T3 group is significantly decrease than other groups. Lymphocytes percentage in T1, T2 and T3 groups were significantly increase than standard value. The serum biochemical alteration in DEHP exposed mice offspring and compared to bon exposed group. The result showed that progesterone in T3 group significantly lower than control group. Consequently, the value of Estrogen in T1, T2 and T3 groups gradually decrease than T0 of offspring

In conclusion, offspring from DEHP-exposed female mice in midge station are particularly sensitive to phthalates poisoning on postnatal growth (birth to weaning and weaning to sexual maturity), hematological and hormonal alterations, as well as organ morphology, in the later life of offspring. Although in this study it was observed that phthalates possess the deleterious effect on the F1 generation of offspring, further experimentation on the multigenerational and transgenerational effects of as will be needed to learn more about the specific health implications and the way that DEHP affects the health of children.

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