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Maternal Di-(2-Ethylhexyl) Phthalate (DEHP) Exposure Impairs Insulin Signal in the Liver and Gastrocnemius Muscles of Female Offspring Rats

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Abstract

Di-(2-Ethylhexyl) Phthalate (DEHP) is a potent endocrine disruptor that is commonly present in consumer products and cosmetics. Exposure to DEHP during gestational and lactational periods can adversely affect glucoregulation and lead to the onset of diabetes in progeny. The liver and gastrocnemius muscles play an important role in regulating glucose metabolism and insulin action. This study was designed to investigate the effect of maternal DEHP exposure on insulin signaling molecules in the liver and gastrocnemius muscles of adult female offspring rats. Rat dams were given DEHP (10 and 100 mg/kg b.wt./day) by oral gavage from gestation day 9 (GD 9) to the end of the lactation period Postnatal Day (PND) 21. On PND 80, female offspring rats in diestrus were euthanized and found reduced body weight, organ (liver and gastrocnemius) weight, and hyperglycemia in DEHP-exposed rats. Western blots revealed a dose-dependent reduction in the expression of Insulin Receptor - (IR), IRS, Akt, and GSK-3β proteins as well as their phosphorylated forms in the liver and gastrocnemius muscles of DEHP-exposed offspring rats. Maternal DEHP exposure reduced the levels of GLUT2 and GLUT4 level in the liver and gastrocnemius muscles, respectively. Liver and renal function markers were dose-dependently increased in the serum of offspring female rats born to DEHP exposed mother during gestation and lactation. Thus, the study revealed that maternal DEHP exposure impaired the expression of insulin signaling molecules in the two important tissues involved in glucose metabolism, the liver and gastrocnemius muscles, suggesting that phthalates exposure during development may contribute to the onset of diabetes in female offspring.

Keywords: DEHP, Gastrocnemius Muscles, Insulin Signaling, Liver, Maternal Exposure

1. Introduction

Di (2-Ethylhexyl) Phthalate (DEHP) is a type of phthalate that finds extensive application in the manufacturing of plastics, cosmetics, medical equipment, and food packaging. As an endocrine disruptor, DEHP can interfere with hormone function in the body. Because DEHP is non-covalently bound to the polymer, it can contaminate the environment and food through product by leaching, resulting in widespread human exposure[1-5]. This exposure can lead to various illnesses affecting the immune system, brain, metabolism, and organ development[6-8]. As DEHP is a lipophilic compound, it can penetrate the placenta, affect the developing fetuses, and target vital organs[9,10]. Experimental studies demonstrated that DEHP has an unpropitious effect on both nutritional and metabolic states[11-14]. In type-2 diabetes, the body becomes resistant to insulin action and diminished insulin response to various target organs like the liver, skeletal muscles, and adipose tissue. Impaired insulin secretion and action contribute together to the development of type 2 diabetes, where insulin resistance leads to pancreatic

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beta cell failure[15]. The genetic and environmental factors are said to contribute mainly to the development of type-2 diabetes[16].

The liver and skeletal muscles are the two vital organs that play a crucial role in glucose homeostasis. In the fasting state, the stored glycogen in the liver is converted into glucose through glycogenolysis and gluconeogenesis, which results in increased glucose in the blood[17]. In the post-prandial state, the excess glucose is converted into glycogen and through glycogenesis stored in the liver and skeletal muscle[18]. Skeletal muscle is the main target tissue for insulin-mediated glucose absorption, and defects in this tissue may lead to diseases like hyperglycemia, type 2 diabetes, etc. People with type-2 diabetes are identified with defects in glucose synthesis and transport in skeletal muscle[19]. Glycogen production and glucose transport are impaired in type 2 diabetics[19]. Cell-specific glucose transporters and insulin receptors aid in the absorption and utilization of glucose. Impairment in insulin action causes an imbalance in hepatic glucose homeostasis, which causes disorders such as hyperglycemia and diabetes.

Several studies have suggested that DEHP exposure may increase the risk of diabetes and insulin resistance, especially if the exposure occurs during critical periods of development. A study by Wang *et al.*, reported that prenatal exposure to DEHP impaired glucose tolerance and insulin secretion in female offspring rats[20]. Another study by Yang *et al.,* demonstrated that phthalates exposure can cause a high prevalence of type diabetes[21]. Phthalates cause diabetes by interfering with the insulin signaling pathway in vital organs that regulate glucose homeostasis. Our previous research explained that DEHP disrupted L6 myotubules viability via oxidative stress and caused a dose-dependent decline in the expression of insulin receptors, Glucose Transporter-4 (GLUT-4), and antioxidant levels, resulting in decreased glucose uptake and oxidation[22]. We also explained that maternal exposure to DEHP during the gestational and lactation period impairs insulin signal transduction in the liver resulting in defective glucoregulatory effects in F_1 male offspring rats[23]. Ovarian steroid hormones are known to regulate hepatic lipid metabolism[24] and control hepatic gene expression[25]. Considering the estrogenic nature of the DEHP, its exposure during development may influence the liver and gastrocnemius muscle function in female rats. Thus, this work was designed to understand the impact of gestational and lactational DEHP exposure on insulin signaling pathway in the liver and gastrocnemius muscles of F_1 female adult rats.

2. Materials and Methods

2.1 Chemicals

Chemicals used in this study were purchased from Amersham Biosciences Ltd; Sigma Chemical, SRL, Mumbai, India; Bio-Rad, USA. Antibodies were purchased from Santa Cruz Biotechnology, CA, USA. HRP- conjugated secondary antibodies were procured from GeNei (Bangalore, India).

2.2 Animals and Experimental Protocol

Adult female Wistar rats (120-130 g), procured from Central Animal House Facility, Dr. ALM Post Graduate Institute of Basic Medical Sciences, University of Madras, Taramani campus, Chennai, India, and maintained in the same facility as per the National Guidelines and Protocols. Rats were fed with standard chow (Lipton India Ltd, Mumbai, India) and water *ad libitum*. This experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC No: 01/29/2015).

During the proestrus phase, female rats were paired (two females and one male) with proven male rats. The following morning, the presence of a copulatory plug or the presence of sperm in the vaginal smear was marked as Ggestational Day-1 (GD-1). Rat dams were exposed to DEHP (10 and 100 mg/ kg. b.wt./day) via oral gavage (GD-9 to PND 21), and control rats received vehicle (olive oil). Every morning, rat dams were weighed, and DEHP doses were calculated according to the body weight. As explained in our previous study[26], the 10 mg/kg/day was based on occupational exposure, which can reach up to 10 to 20 mg/kg/day[27,28], and it is near to No-Observed-Effect Level (NOAEL). Whereas 100 mg/kg/day was selected based on both occupational and medical exposure as it can be as high as 167.9 mg/day[29]. The litter size was culled to six female pups/mother to make equal distribution of DEHP through milk.

2.3 Estimation of Fasting Blood Glucose and Collection of Organs

Animals were fasted overnight at PND 80, and blood was taken from the tail tip to measure Fasting Blood

Glucose (FBG). FBG was estimated using On-Call Plus Blood glucose test strips (ACON Laboratories, Inc., San Diego, CA, USA), and values were mentioned as mg/dL. At the end of the experimental period, rats were anesthetized with sodium thiopentone, blood was collected, sera were separated, and stored at -80°C. To clear blood from the tissues, isotonic saline (20 mL) was used for perfusion. The liver and gastrocnemius muscles were used for the study parameters.

2.4 Assay of Renal and Liver Function Markers in Serum

Liver function markers such as Alkaline Phosphatase (ALP), Aspartate Aminotransferase (AST), and Alanine Aminotransferase (ALT) were assessed in the serum using kit procured from SPINREACT (Girona, Spain). To assess renal function, urea and creatinine levels in the serum were measured according to instructions found in the SPINREAT kit manual.

2.5 Western Blot Analysis

The protein levels of IRβ, pIR$\beta Tyr^{1162/11163}$, IRS-1, pIRS-1ser^{636}, Akt, pAktTyr^{315}, GSK3β, pGSK3βSer^{9}, GLUT2 and GLUT4 (Santa Cruz Biotechnology, Dallas, USA) were analysed by Western blotting. The liver and gastrocnemius muscle were homogenized using Radioimmunoprecipitation Assay (RIPA) lysis buffer with protease inhibitor (Roche, Germany). The homogenate was centrifuged at 12,000 x g for 15 min at 4°C, and supernatant was collected. Using Bovine Serum Albumin as a standard, the protein concentration in the tissue samples was quantified in triplicate. On a 10% sodium dodecyl sulfate-polyacrylamide gel, an equal amount of total protein (50 g/lane) was resolved and further transferred onto a PVDF membrane. (Bio-Rad Laboratories Inc, USA). To block the non-specific sites in the blots, 5% non-fat dry milk powder was used. Blots were incubated in primary antibodies (1:1000 dilution) overnight at 4°C. After washing, the blots were incubated with respective secondary antibodies conjugated with HRP (GeNei, Bangalore, India) in 1:10000 dilution. An enhanced Chemiluminescent reagent (Thermo Scientific, Illinois, USA) was used to detect the antigen-antibody complexes in the blots. The signals were captured and visualized by the ChemiDoc XRS system (Bio-Rad Laboratories, USA). The intensity of the signal was quantified by Quantity One image analysis system (Bio-Rad Laboratories, USA). The same blots were stripped and reprobed with β-actin antibody (1:5000). The intensity of the protein bands was normalized with β-actin.

2.6 Statistical Analysis

Results were given as means ± SEM. To compare the values of control and DEHP treated groups, we used a one-way analysis of variance followed by Students Newman Keul's test (GraphPad Prism version 8, GraphPad Software, California, USA). The difference among groups was considered significant for $p < 0.05$.

3. Results

3.1 Maternal DEHP Exposure Decreased Body Weight, Organ Weight and Increased Fasting Blood Glucose Level

Developmental DEHP exposure decreased the body weight gain in female offspring rats in a dose-dependent

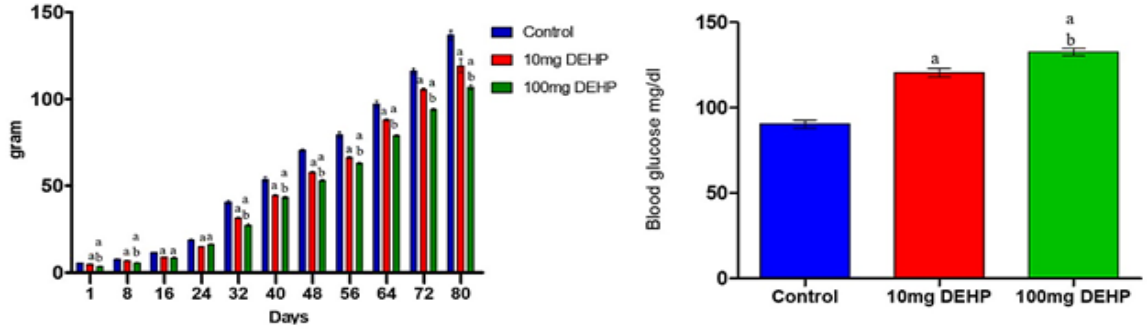

Figure 1. Effect of gestational and lactational DEHP exposure on body weight **(A)** and fasting blood glucose **(B)** of female offspring.

manner (Figure 1A). Fasting blood glucose levels were elevated in rats born to DEHP-exposed mothers (Figure 1B).

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10 mg DEHP.

3.2 Maternal DEHP Exposure Engenders the Risks of Hepatic and Renal Dysfunction in Female Progeny Rats.

Maternal DEHP exposure significantly increased the serum levels of liver function (SGOT, SGPT, and ALP) (Figure 2A) and renal function markers (urea and creatinine) in female offspring rats (Figure 2B).

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10 mg DEHP.

3.3 Maternal DEHP Exposure Impairs the Protein Expression of Insulin Receptor and IRS-1 in the Liver and Gastrocnemius Muscles of Female Progeny Rats

DEHP exposure during gestation and lactation periods impaired glucose homeostasis by altering the insulin signaling molecules. We checked the expression of molecules involved in insulin signal transduction in rat liver and gastrocnemius muscles. Western blot data revealed the decreased level of IR-β (Figure 3A-liver; 6A-GM) and its phosphorylated form (Figure 3B -liver; 6B-GM) was significantly reduced in the DEHP-exposed groups. We further analyzed IRS-1 and its serine phosphorylation and found a dose-dependent decrease in IRS-1protein expression (Figure 3C), whereas the serine phosphorylated form (Figure 3D) was upregulated in the liver of DEHP-exposed female progeny rats.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10mg DEHP.

3.4 Maternal DEHP Exposure Alters the Downstream Molecules of Insulin Signal Transduction in Rat Liver and Gastrocnemius Muscles

We further examined the expression of intracellular signaling molecules involved in insulin signal transduction by western blotting to understand the effects of the decreased IR-β, IRS-1, and their phosphorylated forms in the liver and gastrocnemius muscles of the female offspring rats.

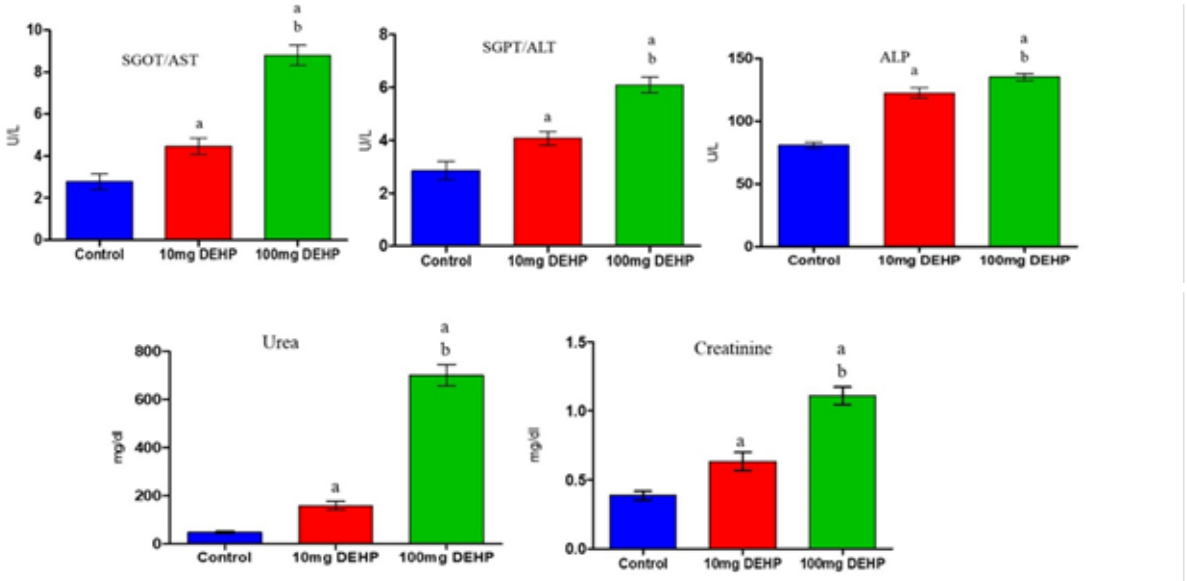

Figure 2. **(A)** Effect of gestational and lactational exposure to DEHP on liver function markers of F_1 female offspring. **(B)** Effect of gestational and lactational exposure to DEHP on kidney function markers of F_1 female offspring.

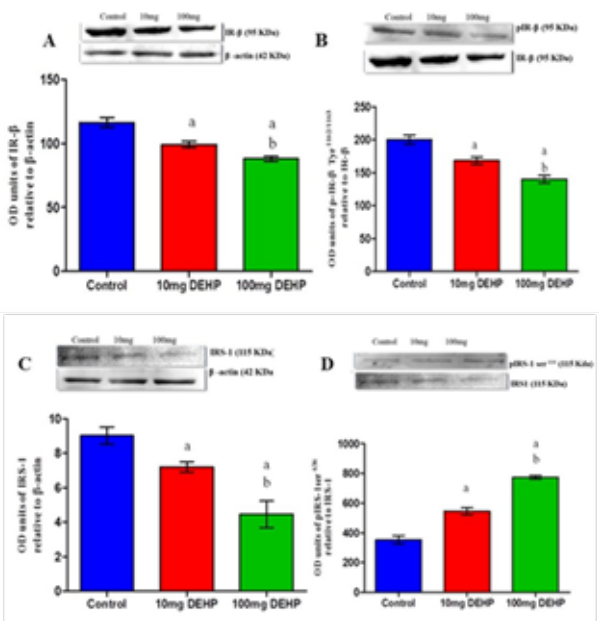

Figure 3. Effect of gestational and lactational exposure to DEHP on insulin receptor, **(A)** p-IR-β Tyr $^{1162/11163}$ **(B),** IRS-1 **(C),** p- IRS-1 ser ^{636} **(D),** proteins in the liver of F_1 female offspring rats.

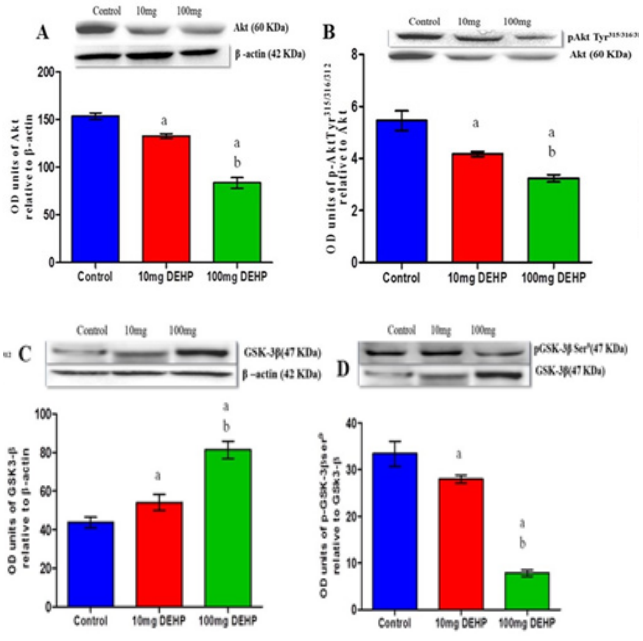

Figure 4. Effect of gestational and lactational exposure to DEHP on Akt **(A),** p-AktTyr$^{315/316/312}$, **(B),** GSK -3β **(C)** and p- GSK-3β ^{ser9} **(D)**, proteins in the liver of F_1 female offspring rats.

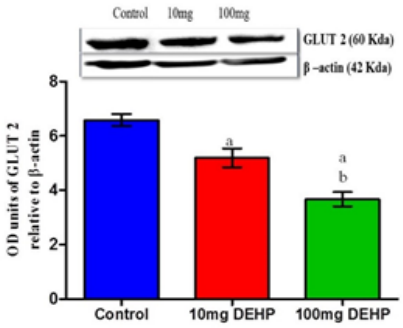

Figure 5. Effect of gestational and lactational exposure to DEHP on GLUT 2 proteins in the liver of F_1 female offspring rats.

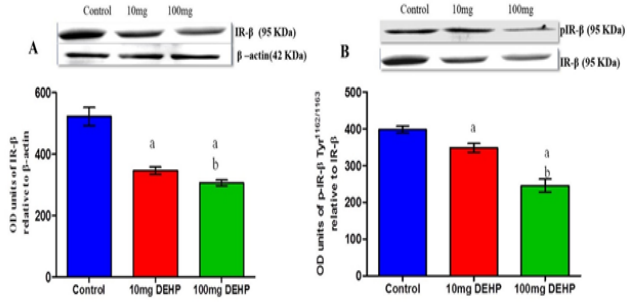

Figure 6. Effect of gestational and lactational exposure to DEHP on Insulin Receptor **(A),** and p- IR-β (Tyr 1162/11163) **(B),** proteins in gastrocnemius muscle of F_1 female offspring.

We observed a dose-dependent decrease in the protein level of Akt (Figure 4A -liver; 7A-GM) and its phosphorylated form (Figure 4B -liver; 7B-GM) in DEHP-exposed offspring rats. Furthermore, it was found that in DEHP-treated rats, GSK-3 was increased (Figure 4C -liver; 7C-GM), and its serine phosphorylation form was dramatically downregulated (Figure 4D -liver; 7D-GM). The protein levels of glucose transporter-2 (GLUT-2) in the liver (Figure 5) and GLUT-4 in the gastrocnemius muscle (Figure 8) were lower in the female offspring rats exposed to DEHP during pregnancy and lactation period.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10mg DEHP.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10 mg DEHP.

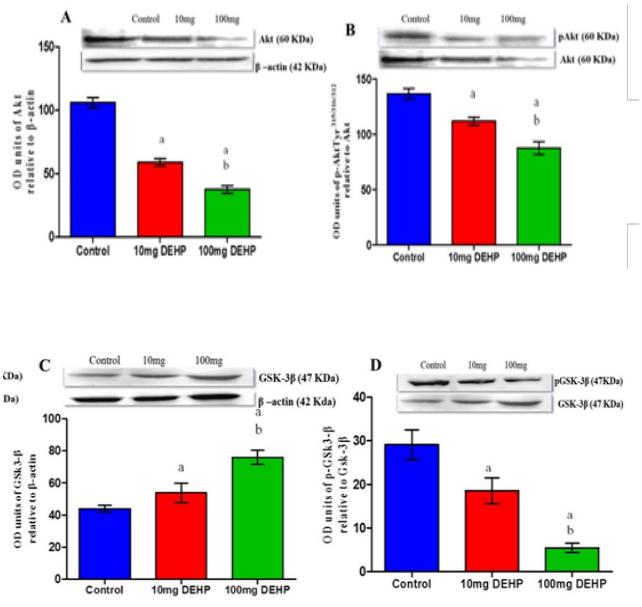

Figure 7. Effect maternal DEHP exposure on Akt (A), p-Akt (Tyr315/316/312) (B), GSK -3β (C) and p-GSK-3β (ser9) (D) proteins in gastrocnemius muscles of F_1 female offspring rats.

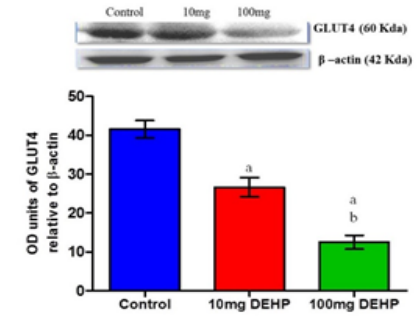

Figure 8. Effect of gestational and lactational exposure to DEHP on GLUT4 protein gastrocnemius muscle of F_1 female offspring.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10 mg DEHP.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10 mg DEHP.

Each value represents Mean ± SEM of 6 rats; $p < 0.05$, a- compared with control; b- compared with 10mg DEHP.

4. Discussion

DEHP is an endocrine-disrupting chemical that can interfere with hormone function and increase the risk of diabetes mellitus. According to the existing reports, DEHP exposure during the developmental period could increase the risk of diabetes in progeny. This study explained the impact of maternal DEHP exposure on the insulin signal transduction in the liver and gastrocnemius muscles of progeny female rats.

To begin with, we evaluated the effects of maternal DEHP exposure on blood glucose levels in F_1 female offspring rats and found a dose-dependent increase in fasting blood glucose levels. Our previous studies explained that maternal DEHP exposure impairs insulin signal transduction and modifies glucoregulatory processes, leading to the development of type 2 diabetes in F_1 male offspring in adulthood[23,26]. Our research suggests that when offspring are exposed to DEHP during their developmental period, they are more likely to get diabetes in adulthood.

Type-2 diabetes is a progressive condition in which the body becomes resistant to insulin action and diminished insulin response to various target organs like the liver, skeletal muscle, and adipose tissue. Insulin acts through its receptor on the cell membrane and maintains blood glucose levels. The decreased insulin receptor protein level is likely to contribute to defective insulin signal transduction and thus, elevated blood glucose levels in DEHP-exposed offspring rats. Insulin signaling is a complex process that involves the activation of IRS1 by insulin. However, IRS1 can also be phosphorylated on serine[6,36] residue by various kinases, which can impair its function and lead to insulin resistance. DEHP has been shown to induce oxidative stress and inflammation in the liver, which can increase serine phosphorylation of IRS1 and reduce insulin sensitivity[30]. The serine phosphorylation of IRS1 is said to have a negative influence on insulin signal transduction[31]. Therefore, maternal DEHP exposure may contribute to the development of metabolic disorders such as diabetes and fatty liver disease by disrupting insulin signaling in the liver.

We measured the levels of insulin receptors and their phosphorylated form, as well as IRS1 and Akt, in the liver and gastrocnemius muscles of F_1 female offspring rats exposed to low and high doses of DEHP. We found that DEHP reduced the expression and phosphorylation of insulin receptors in a dose-dependent manner in both tissues. This implies that the phosphorylation of insulin receptors depends on the number of unphosphorylated

insulin receptors available. Similarly, we observed a dose-dependent decrease in the phosphorylation of IRS1 and Akt, which are downstream mediators of insulin signaling. This was accompanied by a reduction in the total protein levels of IRS1 and Akt, suggesting that DEHP affects their synthesis or stability. Therefore, our results indicate that DEHP impairs insulin signaling in female offspring rats by altering the expression and phosphorylation of key components of this pathway.

GSK-3β is a key enzyme that regulates glycogen synthesis in the liver and muscle. It phosphorylates and inactivates glycogen synthase, the enzyme that catalyzes the formation of glycogen from glucose[32]. In the present study, GSK-3β showed a significant increase, but its phosphorylation at serine[9] decreased in a dose-dependent manner in the liver and gastrocnemius muscle of DEHP-exposed female offspring rats, suggesting that DEHP impaired glycogen synthesis by activating GSK3β and inhibiting glycogen synthase. This may contribute to insulin dysfunction and metabolic disorders caused by DEHP.

GLUT4 is present in the skeletal muscle and adipose tissue, which is a glucose transporter responsible for the uptake of glucose[33,34]. GLUT2 is a bi-directional glucose transporter found in the liver, pancreatic β-cells, kidney, and to some extent in the brain. Levels of GLUT 2 protein in the liver and GLUT4 protein in the gastrocnemius muscle are downregulated in DEHP exposed offspring rats. The decreased GLUT4 in gastrocnemius muscle is suggestive of impaired glucose uptake and its subsequent disposal, which may contribute to elevated blood glucose. Probably, DEHP-induced blunting of insulin action contributed to these changes. The decrease in GLUT2 in the liver is indicative of defective uptake as well as the release of glucose because of impaired glucoregulatory function. In this regard, further studies on gluconeogenesis and glycogenesis would be interesting. The present study suggests that DEHP may influence insulin sensitivity and the uptake of glucose by affecting the expression of genes involved in glucose metabolism. One of the key mechanisms of action of DEHP is through the activation of Peroxisome Proliferator-Activated Receptors (PPARs), which are nuclear hormone receptors that regulate gene expression[35]. DEHP can act as a ligand for PPARs and modulate their transcriptional activity, resulting in various biological effects, including hepatotoxicity and reproductive toxicity.

The liver is the vital organ where the detoxification mechanism takes place. The enzymes in the liver, such as ALP, ALT, and AST are considered liver function markers that involve in protein metabolism. When there is an abnormal level of these enzymes, it represents inflammation, liver damage, and liver dysfunction. These enzymes within the liver cells enter the bloodstream when hepatocytes are damaged[36]. DEHP is considered a toxic substance and affects the integrity of the cell membrane by inducing oxidative stress[37,38]. In this study, there was an increase in all three enzymes in dose-dependent manner in DEHP exposed offspring rats, showing damage in cell membrane and mitochondria. The kidneys are responsible for excreting nitrogen wastes and excess fluids from the body. As a kidney function indicator, urea, creatinine, uric acid, bilirubin, and other protein metabolites are measured. It is considered renal dysfunction when these substances are present in an abnormal amount in the serum or urine[39]. DEHP is a toxic substance that can harm the kidneys. The more DEHP a person is exposed to, the higher the risk of kidney damage[40]. This is shown by the increase in serum urea and creatinine levels, which are markers of kidney function. It is inferred that DEHP exposure during early life can have long-term effects on the liver and kidney function in the offspring.

5. Conclusion

In conclusion, DEHP exposure during gestation and lactation period develop the metabolic disorder in female progeny by disrupting the action of insulin in the liver and gastrocnemius muscles. We also suggest that hepatotoxicity and renal toxicity brought on by DEHP exposure may exacerbate metabolic disorders in the progeny in adulthood.

6. Acknowledgment

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