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# Epigenetic Alteration in Adult Male Testes after Perinatal Exposure to di-(2-ethylhexyl) Phthalate

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## Abstract

The di-(2-ethylhexyl) phthalate (DEHP) is widely used as a plasticizer to increase the flexibility in plastic products. In the present study, the testicular dysfunction in adult male rat offspring after perinatal exposure to DEHP was evaluated. Pregnant rats were administered DEHP (0.75 mg/kg/day by gavage) from gestational day 6 to postnatal day 21. The early life group showed altered expression of several steroidogenesis enzymes in adult male testes compared with control and lifelong groups. Methylation of clusterin promoter region in testes was increased in the early life group compared with the control group. These results indicated perinatal exposure to DEHP may increase DNA damage and result in testicular dysfunction in adulthood.

## Introduction

Among phthalates, di-(2-ethylhexyl) phthalate (DEHP) is commonly used to increase the flexibility of plastic products including food packaging, toys, and medical devices [1]. Due to the wide use of phthalate in consumer products, humans may be exposed to DEHP through ingestion, skin contact, and inhalation during their lifetime. Exposure to DEHP during the developmental period could induce long-lasting adverse effects in adult reproductive function [2,3]. Analysis of DNA methylation, the most well-known mechanism of epigenetic regulation, might help in the understanding of the development of adult onset disease. Previously, DNA methylation was increased in fetuses after maternal exposure to DEHP [4,5]. Hypermethylation and hypo methylation of DNA are associated with the production of oxidative radicals [6]. DEHP induced apoptosis in germ cells by increasing oxidative radicals [7]. Once the production of oxidative radicals is increased, defense mechanisms would start to maintain an adequate homeostasis. To predict the oxidative damage after perinatal exposure to DEHP in adult male rat offspring, clusterin mRNA and protein expression were analyzed. Clusterin has molecular chaperone properties that facilitate inhibition of oxidative stress or cellular oxidative injury, and can be used as a potent cellular marker of oxidative stress [8]. The analysis of clusterin expression and DNA methylation in its promoter region in adult rat offspring might help to understand the biological plausibility of perinatal exposure to DEHP and long-lasting effects on testicular function. Therefore, in the present study, the expression of clusterin and steroidogenic enzymes in rat testes was examined to investigate the adverse effects on testicular function. In addition, the global DNA methylation and methylation of clusterin promoter region was analyzed to evaluate the long-lasting effects on adult testes after perinatal exposure to DEHP.

## Materials and Methods

Pregnant SPF Sprague-Dawley rats (8-week-old) were randomly classified into control group (3 rats) and DEHP-treated early life- and lifelong- groups (3 rats in each group). The administered dosage of DEHP (0.75 mg/kg/day) was approximately 6 times lower than the dosage that caused no adverse effects on testicular toxicity according to the European Food and Safety Authority [9]. Corn oil and DEHP were administered to dams once daily by oral gavage from Gestation Day (GD) 6 to Post-Natal Day (PND) 21. After the weaning period was ended, male offspring were orally administered corn oil (control and early life groups) and DEHP (lifelong group) from PND 22 to PND 69. The number of male offspring in each group was adjusted to 10 on PND 4 to prevent size-induced variability. After the weaning period is ended, male pups were fed standard diet and distilled water *ad libitum* from PND 22 to PND 69. Animals were observed for general toxicity throughout the study period. Male offspring were euthanized with isophorone on PND 70. Blood was collected for analyzing the testosterone and luteinizing hormones, and malondialdehyde

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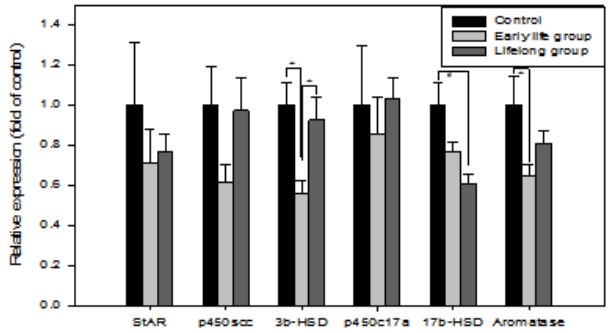


**Figure 1:** The expression of steroidogenic enzymes in testes after DEHP exposure during early life and lifelong period. The results were calculated by the ΔCT method using GAPDH as an endogenous reference. The relative expression levels of genes were calculated as the ratio of gene expression levels in DEHP treated groups relative to those in the control group. Values are means and standard errors of 3 replicates (*p<0.05 and # p<0.01: significantly different from control).

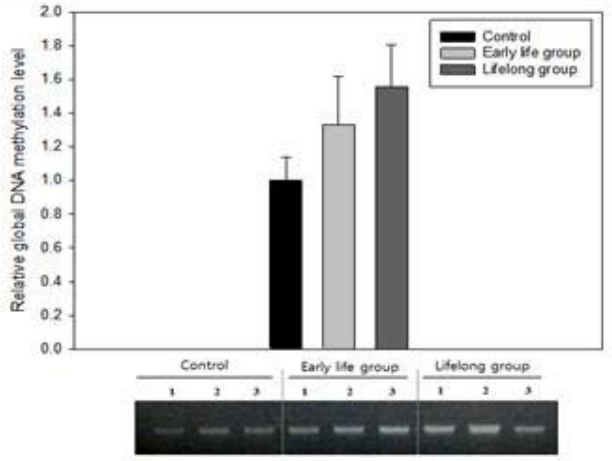


**Figure 2:** Global DNA methylation level in testis after DEHP exposure during early life and lifelong period. The relative expression levels of genes were calculated as the ratio of gene expression levels in DEHP treated groups relative to those in the control group. Values are as mean and standard errors (n=10/group).

levels. Testes was excised and frozen at -80˚C for RNA and DNA extraction. This study was performed in accordance with the Good Laboratory Practice (GLP) guidelines for Animal Experiments from the Korea Testing and Research Institute. Testosterone and luteinizing hormones in serum were measured using a commercial IBL enzyme-linked immuno sorbent assay kit (Endocrine technology Inc, CA, USA). Malondialdehyde levels in serum were analyzed using a Shisheido HPLC/UV system. The effluent was quantified at a wavelength of 532 nm. Total RNA isolation and complementary DNA (cDNA) synthesis were performed as described previously [10]. Each forward and reverse primer for clusterin and steroidogenic enzymes was listed in a previous study [11]. The cycle of real-time PCR reactions consisted of an initial incubation at 95˚C for 10 min, followed by 45 cycles at 95˚C for 10 seconds annealing at 62˚C for 2 seconds, and final extension at 72˚C for 5 seconds. The amplified PCR products were normalized to rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The isolated genomic DNA (2 μg) from testes was separately digested in a total volume of 50 μl with either 20 U of Hap II or Msp I (Takara, Otsu, Japan) at 37˚C for 3 hours. After incubation, the restriction enzymes were inactivated at 70˚C for 20 min

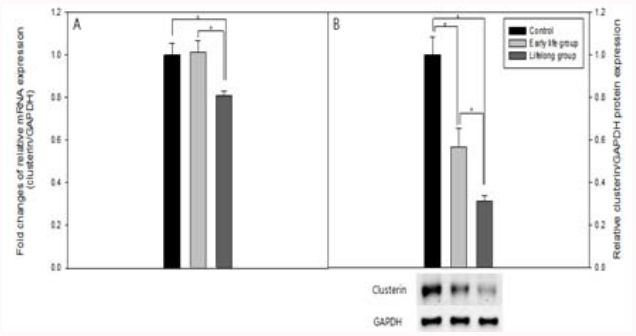


**Figure 3:** Clusterin mRNA (A) and protein (B) expression in testis after DEHP exposure during early life and lifelong period. Expression level of clusterin mRNA was determined by real time RT-PCR and normalized with GAPDH level, and protein was determined by Western blot. Each mRNA was normalized to level of GAPDH-mRNA expression in same preparation. The relative expression levels of genes were calculated as the ratio of gene expression levels in DEHP treated groups relative to those in the control group. Values are means and standard errors (*p<0.05: significantly different from control).

and then stored at -20˚C. The reaction products were analyzed using 1% agarose gel electrophoresis and visualized by ethidium bromide staining under UV illumination. Treatment of DNA with bisulphate for conversion of unmethylated, but not methylated, cytosine to uracil was performed using a bisulphate conversion kit (Epi Tect, Qiagen, Valencia, CA, and USA) according to the manufacturer's instructions. Regarding methylation specific PCR (MSP), the methylated primers for clusterin were 5'-AGTATATTTGATTATTGAA GTTTCGA-3' (forward) and 5'-GACCAAATAAAATAAAACCAAAC GA-3' (reverse). The unmethylated primers for the clusterin promoter region were 5'-AGTATATTTGATTATTGAAGTTTTGA-3' (forward) and 5'-CAACCAAATAAAATAAAACCAAACAA-3' (reverse). All the primers for MSP were designed using the Meth Primer program (http://www.urogene.org/methprimer). The PCR total reaction volume was 30 μl consisting of 10X PCR buffer, 2.5mM dNTP, 0.5 μl of each primer (10 pmol/μl), 5 U Taq DNA polymerase (Promega, Madison, WI, USA) and 100 ng DNA. The amplification conditions for all primers were 95˚C for 5 min followed by 35 cycles of 95˚C for 1 min, annealing at 56˚C for 1min and 72˚C for 1 min, and final elongation at 72˚C for 10 min. The reactions were performed in a Thermocyler (Eppendorf AG, Hamburg, Germany). Each PCR product was analyzed using 1% agarose gel electrophoresis and visualized using ethidium bromide staining under UV illumination. Results are expressed as means ± standard error. The mRNA expression of clusterin and steroidogenic enzymes was compared between the control and DEHP treated groups using the Wilcoxon rank sum test with STATA (version 10.0 Stata Corp LP College Station, TX, USA). P-values less than 0.05 were considered statistically significant.

## Results and Discussion

Serum testosterone levels in the early life group showed significantly higher than the control group (p=0.020) and the lifelong group (p=0.002, Table 1). Luteinizing hormone levels in the early life group tended to lower than the control and the lifelong group. Among the steroidogenic enzymes involved in the production of steroid hormones, the expression of 3β-HSD and aromatase in the early life group were significantly decreased compared with the control group (p=0.017 and p=0.028, respectively; Figure 1). The expression of 3β-HSD in the early life group was significantly lower than the lifelong group (p=0.027). The lifelong group showed

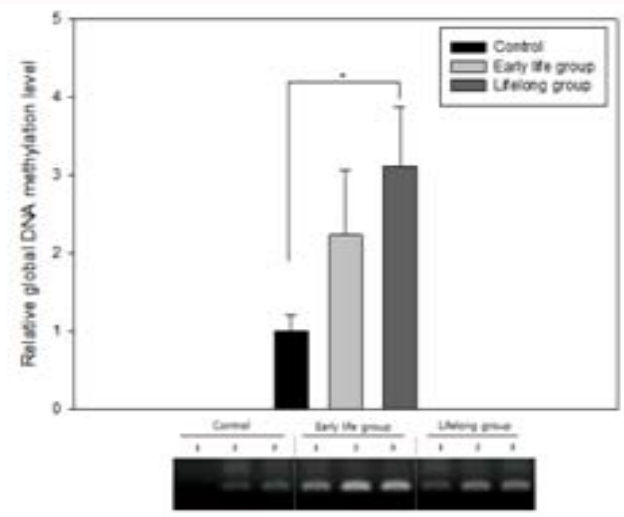


**Figure 4:** Gene specific DNA methylation levels of CpG sites in the promoter regions of clusterin in testis of adult male rats offspring after DEHP exposure during early life and lifelong period. Values are as mean and standard errors (n=10/group; * p<0.05: significantly different from control).

the down-regulated 17β-HSD compared with the control group (p=0.002). It seems that perinatal exposure to DEHP might influence the pathways of steroidogenesis in childhood and left lasting until adulthood. Similar with our study, the decrease of testosterone and the increase of steroidogenic enzymes expression in DEHP treated offspring compared with control [3]. Although they reported the long lasting effects on steroidogenesis in adult male rat offspring, the treated dosages of DEHP were 234 mg/kg/day to 938 mg/kg/day, at least 600 times higher than our study. Thus, it is necessary to consider the effects of low dose exposure of DEHP. Global DNA methylation in testes was increased in the DEHP-treated group compared with the control group (Figure 2). The analysis of DNA methylation is primarily focused on the methylation status of Cytosine-Guanine Pair (CpG) islands. Methylation of these regions generally leads to chromatin condensation and suppression of gene transcription. Similar to our results, maternal exposure to DEHP induced global DNA methylation in testes [4,5]. The results indicated that perinatal exposure to DEHP induced epigenetic alteration in testicular development and resulted in abnormal development of the male reproductive system. The epigenetic alteration in adult male testes might be caused by the imbalance of the oxidative stress and antioxidant status after exposure to DEHP. The increase of oxidative stress due to DEHP induced the apoptosis of testicular atrophy [7]. In the present study, although circulating malondialdehyde levels in serum were similar between the control and DEHP-exposed groups, the clusterin expression in testes was decreased in the early life group and the lifelong period group compared with the control group (p=0.039 and p=0.010, respectively; Figure 3A). In addition, the early life group showed decreased expression of clusterin protein compared with the control group (p=0.049; Figure 3B). The lifelong period group showed significantly decreased expression compared to the control- and the early life- group (both p=0.049). The expression of clusterin promoter DNA methylation in the lifelong period group was increased compared with the control group (p=0.020; Figure 4). Because clusterin has a role in apoptosis protection, the decrease of clusterin mRNA and protein expression in testes after DEHP might represent the disruption of homeostasis between the formation and removal of oxidative radicals.

**Table 1:** The changes of body weight, relative testes weight and circulating hormones in adult male rat offspring. Values are as mean and standard errors (n=10/group).

| Items | Control | Early life group | Lifelong group |
|---|---|---|---|
| Body weight on PND 70 (g) | 437.49±9.18 | 436.01±9.22 | 401.75±14.04 |
| Relative testes weight (%) | 0.82±0.02 | 0.84±0.02 | 0.84±0.02 |
| Testosterone (mg/ml) | 2.51 ± 0.71 | 3.02±0.72[a] | 2.49±0.71[b] |
| Luteinizing hormone (mg/ml) | 2.55±0.25 | 1.93±0.21 | 3.99±0.54 |
| Malondialdehyde (μmol/L) | 0.82±0.07 | 0.98±0.04 | 0.98±0.03 |

a: Significantly different from the control group.
b: Significantly different from the early life group.

## Conclusion

Perinatal exposure to DEHP induced epigenetic alteration *via* the imbalance of oxidative and antioxidant status. The changes of DNA methylation in clusterin promoter region might help to explain the DEHP mediated testicular dysfunction in adult male rat offspring. In the next study, genetic and epigenetic markers will consider to examine the DEHP-induced testicular changes in the subsequent generation.

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