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Changes of Steroidogenic Enzymes and Clusterin in Adult Male Offspring after Early Life Stage Exposure to Low Dose Di (2-Ethylhexyl) Phthalate

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Abstract

Exposure to Di (2-Ethylhexyl) Phthalate (DEHP) during early life stage could increase the risk of male reproductive disorders later in life. However, there has been controversy about the long-term effects of DEHP even lower than the dosage of traditional toxicological studies. This study was examined the association between low dose exposure of DEHP during early life and the alteration of steroidogenesis enzymes and clusterin expression in adult progeny. Pregnant SD rats were administered corn oil (control) and DEHP 0.75 mg/kg/day once daily by oral gavage. Early life stage group was received DEHP from Gestation Day (GD) 6 to Postnatal Day (PND) 21 to dam. Lifelong period group was received from GD 6 to PND 70. Body and organ weight (testis and epididymis), developmental characteristics, histological changes of testis and epididymis, serum hormones (FSH, LH, testosterone and 17β-estradiol), the expression of steroidogenic enzymes and clusterin in testis were examined on PND 70. The expression of 3β-HSD and aromatize in early life stage group, and the expression of 17β -HSD in lifelong period group were significantly decreased compared to control. Clusterin expression in lifelong period group was significantly lower than control groups. In conclusion, perinatal exposure to DEHP at low dose could result in adverse effects on testicular function in adulthood.

Keywords: Di (2-ethylhexyl) phthalate; Low dose; Early life; Steroidogenic enzymes; Clusterin

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Introduction

Di (2-Ethylhexyl) Phthalate (DEHP) is produced by the reaction of 2-ethylhexanol with phthalic anhydride and is the most widely used and produced phthalate [1]. Many consumer products contain phthalates including food packaging, toys, flooring, and wire and cable coating, and they are also used in medical devices including intravenous fluid bags and tubing, blood bags, and dialysis equipment [1]. Once DEHP enters the human body, it is rapidly metabolized by gut lipases, it forms monoesters, and it is further metabolized into oxidative products [2,3]. DEHP and its metabolites have been analyzed in urine specimens to investigate internal exposure [4-6]. Exposure to environmental chemicals during early life is more concentrated than exposure during adulthood because developmental exposure may induce permanent changes. This exposure is associated with the hypothesis of the 'developmental origins of adult health and disease,' which first proposed that poor nutrition in utero might cause metabolic and cardiovascular disease in adulthood [7]. This concept was applied in animal studies to assess the effects on long-term health of DEHP exposure [8,9]. However, it is not yet known which mechanisms in early life exposure influence the adult onset of diseases. The expression of clusterin in testis and epididymis was analyzed to identify the influence of environmental estrogens [10-12]. It is a heterodimeric sulfated glycoprotein that was first identified in ram rate testis fluid [13,14] and that mainly presents in the cytoplasm of Sertoli cells within the testis [14]. It showed molecular chaperone properties that helped to address oxidative stress or cellular oxidative injury [15]. The relationship between DEHP and the clusterin expression in reproductive organs has not been studied. The examination of clusterin in testis might help to explain the reproductive damage in adult males after early life exposure to DEHP because of the oxidative stress stemming from the related mechanisms of DEHP-induced testicular toxicity [16]. Steroidogenic enzymes are involved in the production and secretion of steroid hormones that can affect

Table 1: Sequence-specific PCR primers.

Gene name		Sequence (5'→3')	Accession No.
StAR	F	AGTCATCACCCATGAGCTGG	NM_031558.3
	R	TTCAGCTCTGATGACACCGC	
P450scc	F	CTTTGGTGCAGGTGGCTAG	NM_017286.2
	R	CCGGAAGTGCGTGGTGTTT	
3β HSD	F	TFTGCCAGCCTTCATCTAC	M38178.1
	R	CCTTCTCGGCCATCCTTTT	
P450c17a	F	GTGCAGGGAGAAGTTCGACA	NM 012753.2
	R	CCCCAAAGATGTCTCCCACC	
17β HSD	F	GACCGCCGATGAGTTTGTTA	NM_054007.1
	R	CTTTGGGTGGTGCTGCTGT	
Aromatase	F	GCCTGTCGTGGACTTGGT	NM 017085.1
	R	GGGTAAATTCATTGGGCTTGG	
Clusterin	F	TGCTGCTGACCTGGGACAATG	NM_053021
	R	CCTGGACGGCGTTCTGAATCTC	
GAPDH	F	GGCACAGTCAAGGCTGAGAATG	NM_017008
	R	ATGGTGGTGAAGACGCCAGTA	

the development of male reproductive system. They are classified as the cytochrome P450 (CYP) enzymes including CYP11A and CYP17, and the Hydroxy Steroid Dehydrogenase (HSD) enzymes including 3β-HSD and 17β-HSD [17]. The disruption of steroidogenesis-related gene expressions showed in GD 20 testes after DEHP exposure during fetal period [18]. They suggested the reduction of testosterone might be resulted from the inhibition of basal steroidogenesis. It seems that the interference of the steroidogenic enzymes expression after DEHP exposure during early life stage might affect the production of testosterone and result in abnormal testicular function in their adult period. Therefore, this study was performed to investigate whether exposure to low dose of DEHP during critical developmental periods would induce the adverse effects on the male reproductive system in adult male offspring.

Materials and Methods

Chemicals

Experimental chemicals including Di (2-Ethylhexyl) Phthalate (DEHP) and corn oil were purchased from Sigma-Aldrich Ltd. (Yong-In, Republic of Korea).

Animals

Eight-week-old Pregnant SPF Sprague-Daley rats were purchased from Orient Bio Inc. (Gyeonggi-do, Republic of Korea), and maintained under a controlled environment (22 ± 2 °C; lights on from 06:00 to 20:00 h). To minimize additional exposure of endocrine disruptors, animals were housed in stainless steel cages with wood bedding and provided the distilled water in glass bottle with rubber stoppers surrounded by a steel ring. Animals were provided the standard diet from Purina Korea Inc. (Gyeonggi-do, Republic of Korea) and the distilled water ad labium. The levels of phytoestrogens in the diet did not consider because all animals might expose to the same levels of food-borne phytoestrogens.

Experimental design

This study was performed in accordance with the Good Laboratory Practice (GLP) guidelines for Animal Experiments from the Korea Testing and Research Institute. Pregnant rats were classified into control (corn oil) group and DEHP (0.75 mg/kg/day) treated group. The treated dosage of DEHP 0.75 mg/kg/day was based on the doses of DEHP received by infants who undergo medical procedures (FDA 2002). The number of pregnant rats was 5 rats in control group and 10 rats in DEHP treated group. Each chemical administered once daily by oral gavage to the dam (F0) from Gestation Day (GD) 6 to postnatal day (PND) day 21. The offspring from F0 generation rats were the F1 generation. After the end of lactation, DEHP treated group (F1) divided into early life stage group (n=5) and lifelong period-group (n=5). Early life stage group was administered corn oil after the end of lactation (PND 22 to PND 70), and lifelong period group was continuously treated with DEHP 0.75 mg/kg/day to male pups until PND 70. The male pups were weighed on PND 70, and their blood was collected from aorta for hormone assay. Serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C. The testis and epididymis were immediately excised and weighed. The left testis was frozen at -80 °C for RNA analysis and the caudal part of left epididymis was used for sperm count and motility. The excised and weighed right testis and epididymis were fixed in Bouin's solution for histological studies.

Body eeights and External physical signs

Male pups in F1 generation were recorded the body weight on PND 0, 4, 7 and weekly thereafter until PND 70. Physiological development signs including Ano-genital Distance (AGD), pinna detachment, incisor eruption, nipple retention, eye opening, testes descent, and preputial separation in male pups were observed as previously described [11]. The AGD was divided by body weight for adjustment (AGD/BW $^{(1/3)}$, wAGD).

Histological observation

The fixed testis and epididymis in Bouin's solution for approximately 24 h were washed several times with ethanol (70 %) before embedding in paraffin. Embedded tissue was sectioned at 5 μm and then stained with Hematoxylin, Eosin (H&E). Stained slides were observed under a light microscopy (Olympus Japan Co., Ltd, Tokyo, Japan), and photographs were taken using a Fuji Digital Camera HC 300 Z/CL (Olympus Japan Co., Ltd, Tokyo, Japan).

Hormones in Serum

Separated serum was immediately aliquoted in equal volume (500 μl) into 2 ml of sterile Cryo Vials (Greiner labortechnik, Frickenhausen, Germany), and was kept in a frozen state at -80 °C until analysis. Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), testosterone, and 17β-estradiol in serum were measured using a commercial IBL Enzyme-Linked Immuno Sorbent Assay (ELISA) kit (Endocrine technology Inc, CA, USA) according to the manufacturer's instructions. The absorbance was measured with a plate reader (Tecan Sunrise TW, Salzburg, Austria).

Epididymal sperm count and Motility

The caudal part of epididymis was cut (5 mm) and weighed, and, tubules were dispersed into 5 ml Hank's Balanced Salt solution (Sigma-Aldrich LTD., Yong-In, Republic of Korea) in a plastic petri dish. The petri dish was kept in the incubator at 37 °C in a 5 % CO2 for 10 min. The 1 ml aliquots of sperm suspension solution was mixed in 2 ml of Hank's Balanced Salt solution, then collected and loaded in an 80 μm glass chamber and analyzed using an IVOS automated semen analyzer (Hamilton Thorne Biosciences, Beverly, Mass). The number of epididymal sperm was represented as per gram weight

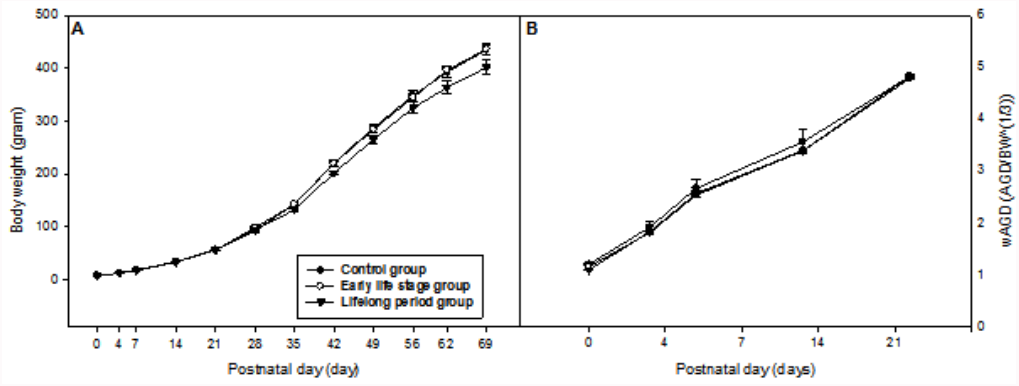

Figure 1: The changes of body weight-adjusted anogenital distance (AGD/BW$^{(1/3)}$, wAGD) in male rats between control and DEHP treated group. Values are means and standard errors.

Table 2: Comparison of serum hormone (FSH, LH, Testosterone and 17β-estradiol) in male rats between control and DEHP treated groups. Data were showed mean and standard errors.

Groups	Hormones			
	FSH (mg/ml)	LH(mg/ml)	Testosterone (mg/ml)	17β-estradiol (pg/ml)
Control	9.475±1.586	2.268±0.395	1.468±0.193	6.807±0.620
Early life stage	8.996±1.012	1.935±0.212*	4.354±1.477	6.543±0.371
Lifelong period	9.124±1.046	3.996±0.548	2.499±0.719	6.533±0.463

*p<0.05

of caudal epididymis tissue. Sperm motility (%) was calculated by dividing the number of motile sperm in sperm count.

Steroidogenic enzymes and Clusterin expression in testis

Total RNA isolation and complementary DNA (cDNA) synthesis was performed as previously described [11]. Clusterin and steroidogenic enzymes were amplified from cDNA with a rat-specific primer (Table 1). Real time Polymerase Chain Reaction (PCR) reactions were performed in duplicate using identical samples of total RNA from testis using Light cycler 2.0 (Roche Applied Science, Mannheim, Germany). Equally alliquoted cDNA (12 mg) synthesized from total RNA were added to a prepared PCR mixture containing a Light Cycler® Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Mannheim, Germany), and each forward and reverse primer for clusterin and steroidogenic enzymes. The cycle of real-time PCR reactions was consisted of an initial incubation at 95 °C for 10 minutes, and followed by 45 cycles at 95 °C for 10 seconds and annealing at 62 °C for 2 seconds, and final extension at 72 °C for 5 seconds. The amplified clusterin and steroidogenic enzymes of PCR products were normalized to rat Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH).

Data analysis

Results are expressed as means ± standard error. The differences between control and DEHP treated group in body weight, hormone levels, epididymal sperm number and motility, clusterin and steroidogenesis enzymes expressions in control- and DEHP treated-groups were evaluated by the Wilcoxon rank sum test using STATA (version 10.0 Stata Corp LP College Station, TX). P-values less than 0.05 were considered statistically significant.

Results

Body eeights and External physical signs

The body weight gain in early life stage showed similar tendency to the control group. Lifelong period group seemed to relatively lower body weight gain from PND 35 to 70 compared to the control group (Figure 1A). The wAGD (Figure 1B) and external physical characteristics including separation of the auricle, eruption of the incisors, separation of the eyelids, nipple retention, descent of the testes, and separation of the prepuce did not differ between control and DEHP treated groups (data were not shown).

Hormones in serum

The levels of FSH, testosterone, and 17β-estradiol in serum in DEHP treated groups were similar with control group (Table 2). In early life stage group, LH levels in early life stage group were relatively lower than control group (p=0.0510).

Epididymal sperm number and Motility

Relative testis and epididymis weights (%) did not show significant change between control and DEHP treated groups (Table 3). The number of sperm in epididymis tended to decrease in early life stage and lifelong period groups compared to the control group. However, sperm motility was similar between control- and DEHP treated groups.

Histologic changes of restis and Epididymis

The progression of spermatogenesis in testis was evaluated (Figure 2A-E). DEHP treated male rats both early life stage and lifelong period did not showed adverse changes of Sertoli cells in seminiferous tubles and Leydig cells in testis compared to control group. Epididymal epithelium and spermatozoa in lumen of epididymal tubule showed similar between control and treatment groups (Figure 2F).

Quantitative expression of steroidogenic enzymes and clusterin

Aromatase and 3β-HSD (T1) expression in early life stage significantly decreased compared to control (p=0.0248 and p=0.0047, respectively; Figure 3A), and reduced 3β-HSD (T3) expression in

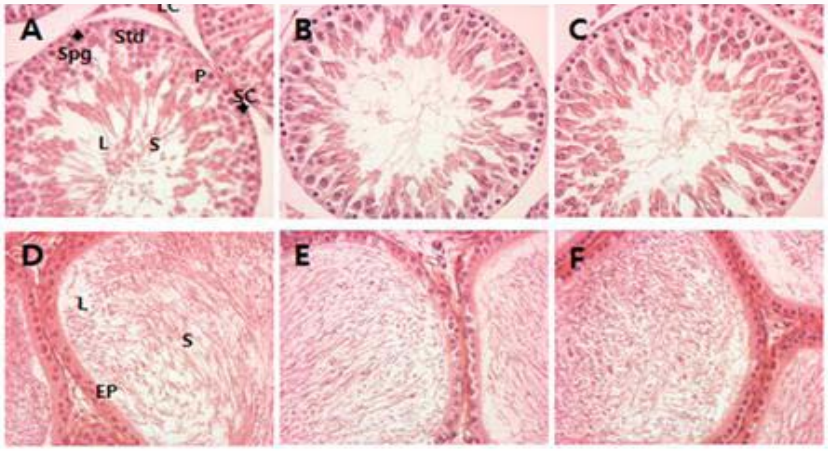

Figure 2: Histological findings in testis and epididymis (×400). The testis and epididymis were stained with hematoxylin-eosin (H&E) and examined under a light microscope (A-C: testis and D-F; epididymis; A and D; control, B and E; early life stage group, C and F; lifelong period group). SC, Sertoli cells; LC, Leydig cells; S, sperm; L, lumen; Spg, spermatogonia; P, primary spermatocytes; Std, spermatids; Ep, epididymal epithelium.

Table 3: Comparison of relative organs (testis and epididymis) weights and epididymal sperm number and sperm motility between control and DEHP treated groups. Values are means and standard errors.

Groups	Relative organ weights (%)		Sperm number (10^6/epi)	Sperm motility (%)
	Testis	Epididymis		
Control	0.790±0.029	0.241±0.004	136.80±26.65	74.69±6.53
Early life stage	0.848±0.020	0.257±0.005	148.40±19.04	74.83±10.95
Lifelong period	0.846±0.024	0.253±0.009	80.80±12.06	61.91±12.77

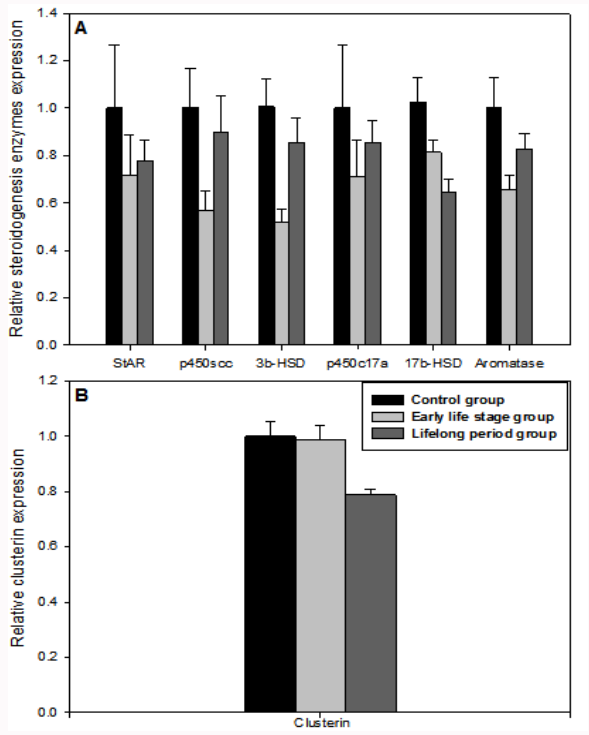

Figure 3: Testicular (A) steroidogenesis enzyme and (B) clusterin mRNA expression. Each mRNA was normalized to level of GAPDH-mRNA expression in same preparation, and the mean of each experimental control (PND 70) was assigned a value of 1.0. Values are means and standard errors (*: p<0.05 and **: p<0.01 compared with controls).

lifelong period showed compared to control (p= 0.0070). Other kinds of steroidogenesis enzymes in early life stage and lifelong period showed similar expression levels between control and DEHP treated group. Clusterin expression in early life stage group showed similar in testis compared to control group. However, lifelong period group was significantly decreased compared to control group (p=0.0036, Figure 3B).

Discussion

This study was performed to investigate whether exposure to DEHP during early life stage could promote epigenetic alterations and result in adverse effects on testicular function in adulthood. The early life group showed lower expressions of 3β-HSD and aromatase in testis compared with the controls. The lifelong period group in the F1 generation showed significantly decreased mRNA expression of 17β-HSD and clusterin in testis compared with the controls. DEHP treatment of 0.75 mg/kg/day resulted in significant reduction of 3β-HSD and aromatase expression in the early life group and of 17β-HSD expression in the lifelong period group; all of these are involved in steroidogenesis (Figure 3A). It has been shown that maternal DEHP treatment between 234 to 1250 mg/kg/day produced a decrease in steroidogenesis-related gene expression in adult male rats [18]. Exposure to di (n-butyl) phthalate 500 mg/kg/day during GD 12 to 21 reduced the expression of the steroidogenic enzymes cytochrome P450 side-chain cleavage, cytochrome P450c17, and steroidogenic acute regulatory protein in male offspring [19]. In addition, decreased testosterone levels in male offspring testis were observed after in utero exposure to DEHP [18,20,21], suggesting that early life exposure to DEHP could suppress testosterone production in the fetal testis and result in reduced circulating levels of testosterone in the adult. However, testosterone levels after exposure to DEHP 0.75 mg/kg/day showed no significant differences in either the early life or the lifelong period groups compared with the control group (Table 2). Testosterone is produced by the Leydig

cells of the testis, which have an important role in testicular descent. DEHP may involve an androgen antagonist that blocks the ability of testosterone to bind to its receptor, resulting in alterations to androgen-dependent tissues. The primary target of DEHP is still unclear; however, DEHP may target the stem cells of the adult-type Leydig cells, which most likely are present in utero, or cells from other organs that subsequently affect testosterone formation by the adult Leydig cells. The decrease in clusterin expression in the lifelong period group might represent the disruption to the male reproductive system after DEHP exposure (Figure 3B). Clusterin was initially studied in the male reproductive system [14] and showed molecular chaperone properties that addressed oxidative stress and cellular oxidative injury [15]. Similar to this study, some endocrine disruptors resulted in the decrease of clusterin expression in testis and epididymis styrene [10-12]. These authors suggested that the down-regulation of clusterin in the reproductive organs might represent the inhibition of spermatogenesis because clusterin secretion is regulated by the Sertoli cells in the testis. Because Sertoli cells are located in the seminiferous tubule and play a role in spermatogenesis, the insufficient testosterone caused by environmental chemicals during early life exposure may result in abnormal development of the male reproductive system [22,23]. Although the statistical changes between the early life and lifelong period groups with the controls were not evident in epididymal sperm number and motility, histological changes in testis and epididymis, or serum hormone levels (Table 2), the decrease in clusterin in testis might have resulted from disrupted masculinization after DEHP exposure. The average daily intake of DEHP in the general population has been estimated at between 3 and 30 μg/kg of body weight/day [24]. However, humans who receive medical treatments might be exposed to higher concentrations of DEHP than the general population [25]. However, patients who receive medical treatment are exposed to higher DEHP levels than the general population because the DEHP that leaches from medical devices could be introduced to the patients [26,27]. The received amount of DEHP in infants (4 kg) was estimated at 3 mg DEHP/kg/day for a period of weeks or months [25]. In addition, some infants who received multiple medical treatments might have received 5 times more DEHP than the tolerable intake levels [25]. Fetus and neonates are not fully developed the endocrine system, thus it could lead to adverse effects on reproduction even low dose of DEHP [28-30].

Conclusion

Perinatal exposure to 0.75 mg/kg/day of DEHP could influence the steroidogenesis pathway in adult male offspring. It seems that exposure to DEHP during early life stage could produce long-lasting effects on male reproductive function. The changes resulted from DEHP could transmit the subsequent generation because of the permanent imprinted-like DNA methylation properties. Further study is needed whether testicular toxicity caused by DEHP could transfer to the next generation or not.

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